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MERCAPTURIC ACID FORMATION IS AN ACTIVATION AND INTERMEDIARY STEP IN THE METABOLISM OF HEXACHLOROBUTADIENE

DIETER REICHERT and SABINE SCHÜTZ

Institute of Pharmacology and Toxicology, University of Würzburg, Versbacher Straße 9, D-8700 Würzburg, Federal Republic of Germany

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Abstract—^{14}C-hexachlorobutadiene (HCBD), a mutagenic and nephrocarcinogenic pollutant, was administered by oral gavage of 100 mg/kg to female rats, and the radioactivity in 24 hr urine pooled. The average amount of radioactivity recovered in urine was 5.4% of the total ^{14}C-activity ingested. Solvent extraction, high performance liquid chromatography (HPLC), radio gas chromatography and gas chromatography/mass spectrometry were used for separation and identification of metabolites. After solvent extraction and HPLC four fractions were separated containing 1%, 5%, 15% and 80% of radioactivity. In the 80% fraction one metabolite was identified after derivatization and comparison with the authentic compound as the mercapturic acid of HCBD (*N*-acetyl-*S*-1,1,2,3,4-pentachlorobutadienyl)-L-cysteine). The mercapturic acid accounts for 10% of the urinary ^{14}C-activity.

In a first attempt the mutagenic potential of the mercapturic acid was determined on *Salmonella typhimurium* TA 100 with and without metabolic activating S9 mix. In the presence of S9 mix the mercapturic acid exerts a strong mutagenic effect which proved to be about 80 times higher than that of HCBD. The results identify the formation of the mercapturic acid via direct glutathione conjugation as an activating and intermediary step in the metabolism of hexachlorobutadiene.

Hexachloro-1,3-butadiene (HCBD) is a well known environmental contaminant [1]. This compound is of high toxicological interest as it acts as an allergen [2], mutagen [3, 4] and nephrocarcinogen [5]. The distinct organotropic properties suggest a metabolic activation mechanism which differs basically from other chemically related compounds. This assumption is supported by the finding that the following main metabolites of HCBD are exclusively conjugation products: the urinary metabolites pentachlorobutadienyl-mercaptoacetic acid and pentachlorobutadienyl-methylthioether [6] and pentachlorobutadienyl-glutathione in the bile of rats [7]. Glutathione conjugation serves to detoxify a large number of xenobiotics [8]. However, in the metabolism of HCBD the conjugation obviously promotes metabolic activation [7]. No metabolite of HCBD has been identified so far which would indicate a direct oxidation reaction at the two double bonds of the molecule.

The enzyme β-lyase, which cleaves the C—S bond in the HCBD–cysteine conjugate to form the thiol [9] plays a key role in the activation sequence of HCBD. The thiol is expected to be rather unstable and undergoes further metabolism to form the ultimate reactive intermediate. The question arises, whether the HCBD–cysteine conjugate is quantitatively metabolized by β-lyase to the thiol (and partly deaminated to the *S*-pentachlorobutadienyl-mercaptoacetic acid) or whether it is also a substrate of the *N*-acetyltransferase to form the mercapturic acid. The mercapturic acid has not been identified so far, but has been predicted as an inactivating pathway [6]. In order to establish the existence of this pathway, to identify the mercapturic acid and to quantify its renal excretion, as well as to attempt to describe the toxicological relevance of its formation, we carried out further metabolism studies with rats and compared the mutagenic potentials of HCBD and the mercapturic acid by use of *Salmonelle typhimurium* TA 100 assays.

* Abbreviations used: GC, gas chromatography; HCBD, hexachloro-1,3-butadiene; HPLC, high performance liquid chromatography; MS, mass spectrometry; mercapturic acid of HCBD, *N*-acetyl-*S*-(1,1,2,3,4-pentachlorobutadienyl)-L-cysteine.

MATERIALS AND METHODS

Chemicals

HCBD. This was purchased from Merck (Darmstadt, F.R.G.). The product was of analytical grade and was subjected for this study to a purification procedure as described previously in detail [3]. The purity of HCBD used in this study exceeded 99.5% as analyzed by GC.

^{14}C-*HCBD* was purchased from Hoechst (Frankfurt, F.R.G.) and had a specific radioactivity of 38.85 MBq/mmol (1.05 mCi/mmol). Its radiochemical purity exceeded 99%, as shown by radio-GC.

Mercapturic acid. *N*-acetyl-*S*-(1,1,2,3,4-pentachlorobutadienyl)-L-cysteine was prepared by addition of 16.3 g (100 mmole) *N*-acetyl-L-cysteine and 4.6 g (200 mmole) sodium in small portions to 400 ml dry ethanol at 0°; this solution was added dropwise to a solution of 26.2 g (100 mmole) HCBD in 250 ml dry ethanol, and the reaction mixture then was stirred for 90 min at 20°. The solvent was evaporated *in vacuo* and the solid residue dissolved in water. This aqueous phase was extracted repeatedly

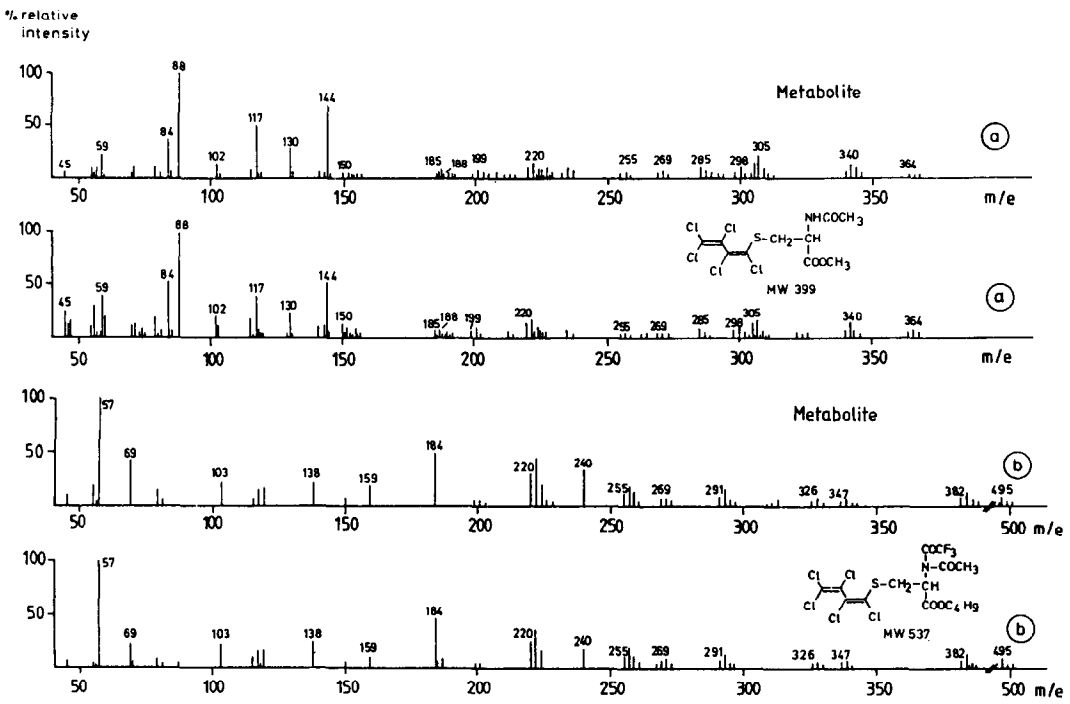

Fig. 1. Mass spectra of a HCBD metabolite and two authentic reference compounds (methyl ester (a) and trifluoroacetylated butylester (b)) after two different derivatization procedures.

with petroleum ether to remove unchanged HCBD and then adjusted to pH 4 with acetic acid. The resulting precipitate was collected by centrifugation. Purification of the crude product by column chromatography on silica gel with methanol/methylene chloride 3:7 v/v yielded colourless crystals. The purity exceeded 99%, as shown by GC. U.v. spectrum (methanol): λ_{max} 218 nm, $\varepsilon = 19800$, λ_{max} 283 nm, $\varepsilon = 10500$. For GC/MS either the methyl ester was formed with 3 N methanolic HCl (60°, 20 min), or the trifluoroacetylated butyl ester with 3 N butanolic HCl (80°, 15 min) and trifluoracetic anhydride. For MS see Fig. 1.

Animals and treatments

Female Wistar rats (180–220 g, Institut für Versuchstierzucht, Hannover, F.R.G.) were used. Single doses of 100 mg/kg ^{14}C-HCBD, dissolved in tricaprilin (2 ml), were administered by gastric intubation between 9:00 and 10:00 hr. Pairs of rats were placed immediately after dosing in all-glass metabolism cages designed for the separate collection of urine and feces. A constant flow of air (500 ml/min) was drawn through the cages. Standard diet (Altromin) and tap-water were supplied *ad libitum*; 72 hr after HCBD gavage, animals were killed by cervical dislocation.

Isolation of urinary metabolites

Measurement of radioactivity was performed in aliquots of the liquid samples with Rotiszint 22 liquid scintillator medium (Roth, Karlsruhe, F.R.G.) on a Packard TriCarb 2650. Metabolites were isolated from 24 hr urine. This was deproteinized with two volumes of ice-cold ethanol and centrifuged at 9000 *g* for 30 min. The supernatant was evaporated *in vacuo* at 30° to one third and extracted about ten times with 30 ml of ethyl acetate at pH 6–7. The organic layers were combined, evaporated at ambient temperature to a volume of 1 ml and subjected to a SepPak column (C-18); methanol was the eluent. The methanolic eluate was again concentrated *in vacuo* (1 ml) and chromatographed on a reversed phase HPLC column (8.0 × 250 mm LiChrosorb RP-18, particle size 10 μm, Knauer, Berlin, F.R.G.). A linear solvent gradient from 5% methanol in water to 100% methanol over a time period of 45 min was used. HPLC fractions were combined according to the radioactivity peaks and evaporated to dryness. The residue was then either methylated with an ethereal solution of diazomethane or butylated with 3 N butanolic HCl and subsequently trifluoroacetylated with trifluoroacetic anhydride. The samples were analyzed by radio-GC and GC/MS after derivatization. The separation conditions of the radio-GC have been described previously [6]; GC/MS was performed on a Finnigan 4510 GC/HSIDs. The mass spectra were recorded at an electron energy of 70 eV.

Mutagenicity testing

Salmonella typhimurium strain TA 100 was used; the characteristic properties of this strain were checked regularly (u.v. and crystal violet sensitivity, ampicillin resistence, mutability by u.v. and benzo(*a*)pyrene). "Overnight" cultures were grown in Oxoid Nutrient Broth from Aroclor-1254 induced male Wistar rats and contained 27 mg protein per ml. S9 mix was composed according to Maron and Ames [10], the S9 concentration in the S9 mix was 10% (mercapturic acid) or 30% (HCBD). HCBD is

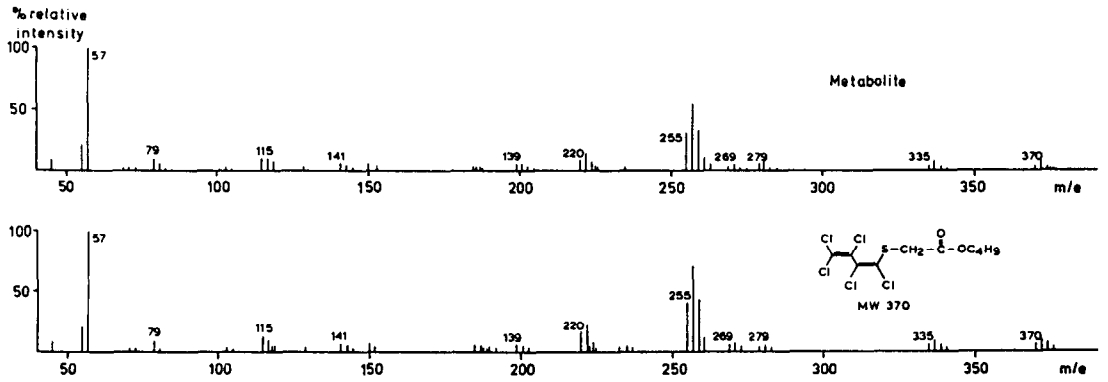

Fig. 2. Mass spectra of a HCBD metabolite and the corresponding authentic reference compound (butyl ester).

mutagenic only in the presence of this fortified S9 mix condition [3].

The mutagenicity assays were carried out by preincubation at 37° of 0.1 ml bacterial culture, 500 μl activation system and 20 μl of a solution of HCBD in dimethylsulfoxide, or a solution of the mercapturic acid in methanol. After preincubation, 2 ml of top agar containing histidine and biotin were added and the mixture was plated on Vogel-Bonner E medium [10]. After 2 days of incubation revertant colonies were counted using an automated colony counter; the raw counts were corrected for overlapping colonies by means of a computer program [11].

RESULTS

Isolation and identification of the mercapturic acid

Metabolites were isolated from the pooled 24 hr urine of two rats after a single p.o. administration of 100 mg/kg ^{14}C-HCBD. The majority of radioactivity was recovered after 72 hr from feces (60%). The average amount of ^{14}C-activity recovered in urine after 24 hr was 5.4% of the total radioactivity. After solvent extraction with ethyl acetate at neutral pH the organic layer contained more than 50% of the urinary radioactivity. Chromatography on a reversed phase HPLC column (RP-18) yielded four fractions of radioactivity: 1, 1%; 2, 5%; 3, 80%; 4, 15%. In fractions 1 and 2 no metabolites were found under the described separation and derivatization conditions. Radio-GC and GC/MS revealed four metabolites after derivatization of fraction 3.

The mass spectrum of one metabolite from fraction 3 shows the typical isotope pattern of five chlorine atoms and corresponds in GC retention time and mass spectrum with the authentic mercapturic acid. In Fig. 1 the mass spectra of the methyl ester and of the trifluoroacetylated butylester of this metabolite and the authentic compounds are shown. This metabolite accounts for *ca* 10% of the urinary radioactivity.

The mass spectrum of another major metabolite of fraction 3 is shown in Fig. 2. Again this spectrum is characterized by the presence of five chlorine atoms; cochromatography and mass spectrum of the authentic compound prove that this metabolite is *S*-pentachlorobutadienyl-mercaptoacetic acid. This metabolite has been isolated previously using different separation conditions and methylation [6].

In the most lipophilic HPLC fraction 4 we isolated two metabolites; however, as yet no unequivocal structure could be assigned to them. From the fragmentation pattern unconjugated metabolites can be assumed.

Mutagenicity of the mercapturic acid of HCBD

To establish whether the conjugation reaction of HCBD with glutathione is an activation step in the metabolism of this compound, the mutagenic potential of the mercapturic acid was investigated with *Salmonella typhimurium*. This metabolite exerts a strong mutagenic effect in *Salmonella typhimurium* TA 100 in the presence of S9 mix (Fig. 3). The mutagenic effect of the mercapturic acid is about 80

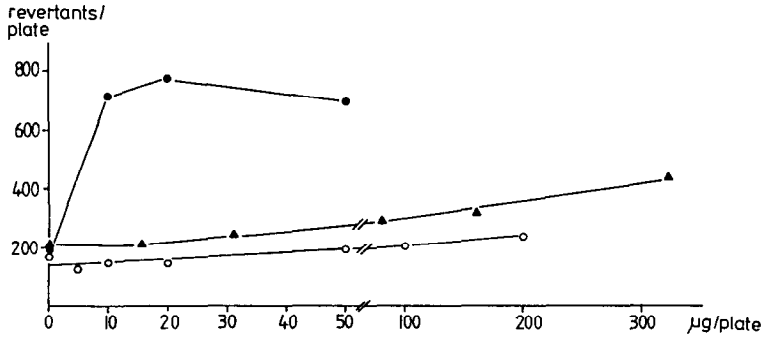

Fig. 3. Mutagenicity of the mercapturic acid with (●) and without (○) S9 mix and HCBD with (▲) S9 mix. HCBD is mutagenic only under fortified conditions in the presence of S9 mix with an increased protein content (see methods).

times greater than that of HCBD, despite the fact that in the assay with HCBD an increased protein content was used.

DISCUSSION

The identification of the mercapturic acid as an ultimate urinary metabolite of HCBD completes the reaction pattern of the HCBD–cysteine conjugate. The available evidence suggests that this conjugate is the substrate of at least three different enzyme systems (Fig. 4): 1, of the enzymes decarboxylase, dehydrogenase and deaminase to form the carboxymethylthio derivative of HCBD; 2, of *N*-acetyltransferase to form the corresponding mercapturic acid; and 3, of β-lyase to form the pentachlorobutadienyl-thiol. The carboxymethylthio derivative has been found recently as an ultimate urinary metabolite [6] This degradative pathway contributes in all probability to an irreversible inactivation of the conjugate. In analogy, a comparable compound has been found in the metabolism of chlorinated ethylenes (e.g. vinyl chloride and vinylidene chloride) in the form of the thiodiglycolic acid which is the main urinary metabolite of these structurally related aliphatics [12, 13]. The third pathway, which leads to the thiol, is suggested as a crucial activation step in HCBD metabolism and is discussed elsewhere [6, 7].

The mercapturic acid pathway found in this study appears to serve, as generally accepted, as a detoxication mechanism. This protective mechanism is confirmed by numerous reports [8, 14]. It has been shown by Tateishi and coworkers [15] that a mercapturic acid can remain unchanged as an ultimate metabolite or can also be the substrate of an *N*-deacetylase. The assumption of deacetylase activity in HCBD metabolism would influence pharmacokinetic and pharmacodynamic parameters. The recycling of the HCBD mercapturic acid to the cysteine conjugate can increase both the half-life of the excretion of metabolites and the biological reactivity. In order to provide evidence of the metabolic conversion of the mercapturic acid, we studied its mutagenic response with the *Salmonella typhimurium* TA 100 strain with and without metabolic activating S9 mix. In the absence of S9 mix the mercapturic acid is not mutagenic, whereas in the presence of S9 mix its mutagenic response is 80-fold that of HCBD. These findings unequivocally support a metabolic recycling of the mercapturic acid and we can assume that an activation involves the *N*-deacetylase and in a subsequent step the β-lyase. Both enzymes are constituents of the S9 mix. Studies are in progress to determine the specific requisites for the activation of the mercapturic acid [16].

The suggestion that the mercapturic acid of HCBD is recycled by an *N*-deacetylase in the tubulus cell of the kidney to the cysteine conjugate explains the relatively low amount of the mercapturic acid in the urine of rats. In our experiments *ca* 10% of the urinary radioactivity can be ascribed to the mercapturic acid. In addition this reactivating pathway would account for a multitude of observed urinary metabolites and would designate the thioether cleavage, catalyzed by the enzyme β-lyase, as the rate-determining step in the metabolism of HCBD. The ultimate excretion of the mercapturic acid as an inactive urinary metabolite is a minor pathway. These findings should be considered in regard to therapeutic strategies which recommend gavage of *S*-containing antidotes such as cysteine and/or ace-

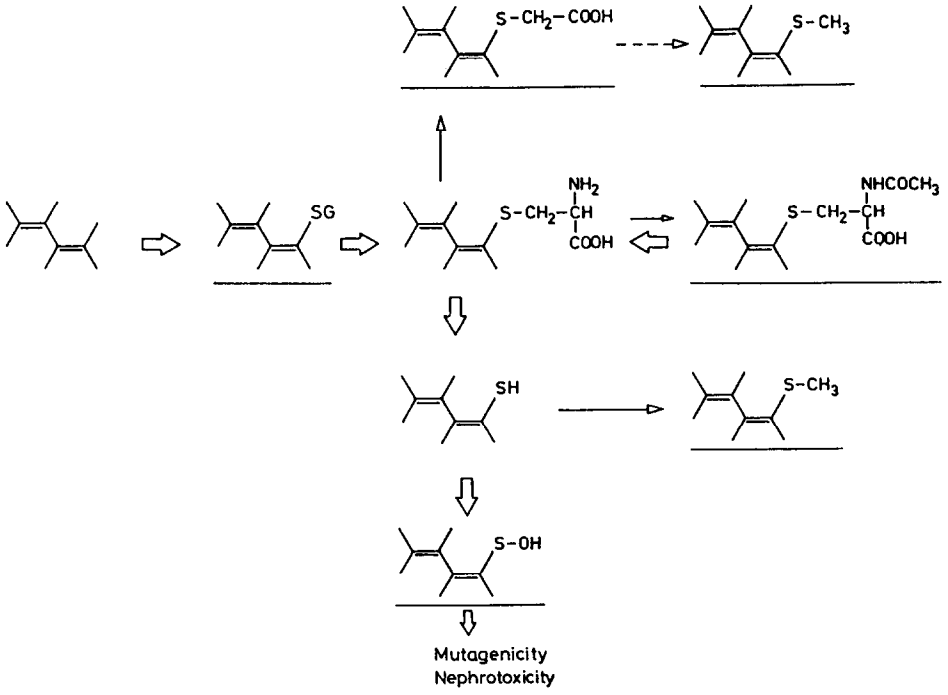

Fig. 4. Proposed metabolic activation reaction of HCBD and of its mercapturic acid: broad arrows mark the activation, small arrows the deactivation steps. Identified metabolites are underlined.

tylcysteine to treat intoxication with compounds which are precursors of mercapturic acids. Therapy of a HCBD intoxication with these antidotes could increase rather than decrease the toxic effects of HCBD.

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