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MERCAPTURIC ACID FORMATION AND ENZYME-CATALYZED CONJUGATIONS WITH GLUTATHIONE IN PIGEONS

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SUMMARY

Pigeons are able to metabolize 3,4-dichloronitrobenzene (DCNB) and 2,3,5,6-tetrachloronitrobenzene (TCNB). The main metabolic route for DCNB is reduction of the nitro group and mercapturic acid is a minor metabolite. TCNB is converted to mercapturic acid. High-speed supernatant of pigeon liver contains enzymes which catalyze the conjugation of glutathione with DCNB and 2,4-dinitrochlorobenzene. Nitrite ions are enzymatically released from TCNB and 4-nitropyridine *N*-oxide during incubation with glutathione. Experiments with inhibitors are included.

INTRODUCTION

Many chlorinated mononitrobenzenes are excreted by rabbits in the urine as mercapturic acids (*S*-aryl-*N*-acetylcysteines)[1]. Studies with high-speed supernatants of rat liver revealed the presence of glutathione *S*-aryltransferase[2], which catalyzed the reaction of glutathione with the foreign compound as the initial step in mercapturic acid synthesis[2,3]. The reaction involves a replacement of the Cl atom or the NO_2 group. Distribution studies of glutathione *S*-aryltransferase in various invertebrate species[4] and in locusts and other insects[5], indicated large differences in the amount of this enzyme. Little is known of mercapturic acid formation in birds. A major interest of this laboratory is the influence of pesticides on wild life. This study forms part of an investigation of detoxication mechanisms in birds. Two chloronitrobenzenes were chosen for *in vivo* experiments to determine the qualitative aspects of their metabolic patterns since these substances are known to be precursors of mercapturic acids in rabbits. Mercapturic acid formation is initiated by the replacement of Cl4 (DCNB) and of NO_2 (TCNB) by glutathione. For the *in vitro* experiments, two additional compounds were examined, 2,4-dinitrochlorobenzene (Cl replacement) and 4-nitropyridine *N*-oxide (NO_2 replacement). Some experiments with competitive substrates are included.

Abbreviations: DCNB, 3,4-dichloronitrobenzene; TCNB, 2,3,5,6-tetrachloronitrobenzene.

MATERIALS AND METHODS

Animals and treatment

Pigeons from which food had been withheld for 16–18 h were given doses of DCNB (1000 mg/kg body wt.; 10 animals) and of TCNB (1000 mg/kg body wt.; 10 animals) administered into the proventriculus. During the first few hours following administration the pigeons vomitted frequently. The excreta were collected at regular intervals, especially on the first day, avoiding contamination with the regurgitated material as far as possible, and were stored at 0–5°. Collection of excreta was continued for 3 days and the excreta were worked up to crude solids or solutions in the day following collection.

Reference materials for identification of the pigeon metabolites were obtained by isolation of the metabolic products from rabbit urine after oral administration of 1000 mg/kg body wt. of each compound. Both pigeons and rabbits were given food and water ad libitum after treatment.

Materials

DCNB, 2,4-dinitrochlorobenzene and glutathione were obtained from British Drug Houses; TCNB, 4-nitropyridine *N*-oxide and sulphobromophthalein (sodium salt) came from Koch-Light Laboratories; 3,4-dichloroaniline was prepared by reduction of DCNB by iron powder[6] (m.p., 71.5°); *S*-(2-chloro, 4-nitrophenyl)-glutathione was prepared as described by BOOTH, BOYLAND AND SIMS[7]; *S*-(2,4-dinitrophenyl)-glutathione·1 H_2O was synthesized by the method of BOST, TURNER AND MORTON[8] for mercaptans (1.0 g glutathione, dissolved in 6 ml 0.5 M NaOH, was added at once to 30 ml hot ethanol, containing 2.0 g 2,4-dinitrochlorobenzene, and the mixture was left on the steambath for 20 min; after addition of 20 ml ethanol, the precipitate was collected from the cold solution and recrystallized twice from boiling water; the crystals were dried over NaOH *in vacuo* (yield, 850 mg); m.p., 192–194° (decomp.)[9]; 3,4,3′,4′-tetrachloroazoxybenzene (m.p., 138°) and *N*-acetyl-*S*-(2-chloro-4-nitrophenyl)-L-cysteine (m.p., 188°) were isolated from the urine of rabbits given DCNB[10]. 2,3,5,6-Tetrachloroaniline (m.p., 108°), 4-amino-2,3,5,6-tetrachloroaniline (m.p., 184°) and *N*-acetyl-*S*-(2,3,5,6-tetrachlorophenyl)-L-cysteine (m.p., 211°) were isolated from rabbit urine after treatment with TCNB[11].

Methods

Isolation and identification of metabolites of DCNB: The excreta were continuously extracted with acetone. The extract was diluted with water and prepared according to BRAY, JAMES AND THORPE[10]. The metabolites were identified by paper chromatography and by mixed-melting-point determinations, if sufficient material was available.

Isolation and identification of metabolites of TCNB: The excreta were dispersed in dilute NaOH solution (pH 10–11) and filtered after 2 h. The metabolites were isolated from the acidified extract and identified by determination of mixed melting points and paper chromatography.

Liver preparations and *in vitro* studies: Livers were homogenized in 4 vol. of 0.1 M pyrophosphate, pH 7.4, and the homogenate was centrifuged at 70 000 × *g* for 1 h. If not used immediately, the clear supernatant was stored at —20°. The enzyme activity towards DCNB and 2,4-dinitrochlorobenzene was determined by measuring

the amount of the glutathione derivatives according to BOOTH, BOYLAND AND SIMS[7] and COHEN, SMITH AND TURBERT[5], respectively. The enzymic conjugation of TCNB and of 4-nitropyridine *N*-oxide with glutathione was measured by determining the release of nitrite ions[12], making corrections for spontaneous release. The protein content of the supernatant was determined by the biuret reaction. K_m and K_i values were obtained by the graphical method of DIXON[13]. For further interpretation of Figs. 2, 3, refer to WEBB[14].

RESULTS

Experiments in vivo

DCNB: 3,4-Dichloroaniline and 3,4,3′,4′-tetrachloroazoxybenzene were isolated from the excreta. Traces of mercapturic acid were present and identified by paper chromatography. Chlorinated aminophenol, mentioned as a probable metabolite by BRAY, JAMES AND THORPE[10], was not detected.

TCNB: Mercapturic acid was isolated from the excreta, but 2,3,5,6-tetrachloroaniline and 4-amino-2,3,5,6-tetrachlorophenol were not detectable on paper chromatograms.

Experiments in vitro

Specific activity in the 70000 × *g* pigeon liver supernatant: The specific activity was determined with 4 substrates: DCNB (1.25 mM) and glutathione (3.75 mM); 2,4-dinitrochlorobenzene (1.0 mM) and glutathione (5.0 mM); TCNB (0.1 mM) and glutathione (6.0 mM); 4-nitropyridine *N*-oxide (0.5 mM) and glutathione (6.0 mM). The results given in Table I refer to 10 pigeon liver supernatants, males and females being equally distributed. There was no statistical difference between the sexes (Wilcoxon test). For comparison, the activity of a single rat liver supernatant is given.

TABLE I

GLUTATHIONE *S*-ARYLTRANSFERASE IN 70000 × *g* PIGEON LIVER SUPERNATANT FOR DIFFERENT SUBSTRATES

Enzyme activities were measured as described in METHODS. The values for a single rat liver preparation (70000 × *g*) are included for comparison.

	*μmoles product/h per mg protein** (*mean ± S.D.*)		
	Rat	*Sex*	*Pigeon*
3,4-Dichloronitrobenzene	2.3	♀	0.17 ± 0.03
		♂	0.15 ± 0.03
2,4-Dinitrochlorobenzene	3.3	♀	5.0 ± 1.1
		♂	4.2 ± 1.7
2,3,5,6-Tetrachloronitrobenzene	22.4	♀	40.5 ± 11.6
		♂	32.0 ± 10.2
4-Nitropyridine *N*-oxide	200.0	♀	75.2 ± 26.9
		♂	76.6 ± 25.1

* The total protein per 70000 × *g* supernatant for rat and pigeon amounts to 1040 mg and 1680 mg on the average. To evaluate the total enzyme activity per liver specific activities have to be multiplied by 1040 and 1680 for the rat and the pigeon, respectively.

K_m values

Both rat and pigeon liver supernatants have the same K_m values for DCNB *viz.* $1.0 \cdot 10^{-3}$ M as shown in Fig. 1. No K_m values could be obtained for 2,4-dinitrochlorobenzene, since the curves were alinear. A similar result was recorded by COHEN, SMITH AND TURBERT[5], using insect preparations. Because of the particular reaction

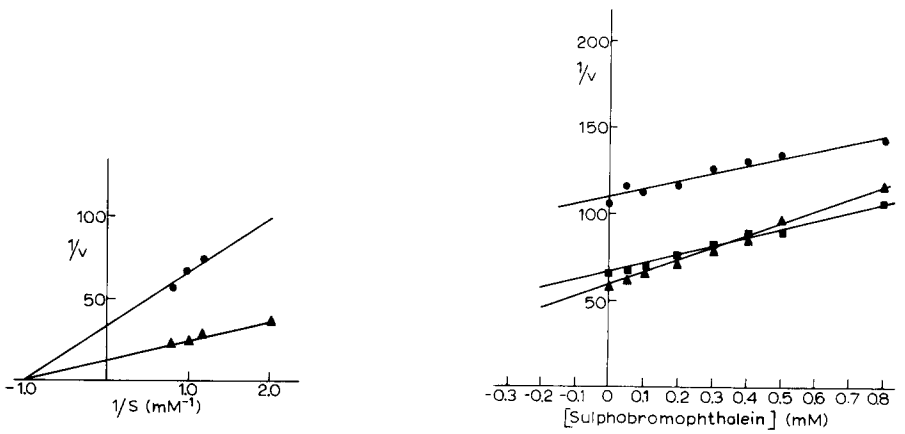

Fig. 1. K_m (DIXON[13]) plot for 3,4-dichloronitrobenzene for pigeon liver supernatant (●), respectively rat liver supernatant (▲). Reaction mixtures, containing glutathione (3.75 mM) and supernatant in 0.1 M pyrophosphate buffer (pH 8.0), were incubated at 37°. The enzyme activity, v, was measured by the increase in the absorbance at 344 mμ (ref. 7).

Fig. 2. Uncompetitive and substrate inhibition type WEBB[14] plots obtained by regarding 3,4-dichloronitrobenzene (●, 0.50; ■, 1.00; ▲, 1.25 mM) as the substrate and sulphobromophthalein as the inhibitor. Reaction mixtures containing glutathione (3.75 mM) and pigeon liver supernatant in 0.1 M pyrophosphate buffer (pH 8.0) were incubated at 37°. The enzyme activity, v, was measured by the increase in the absorbance at 344 mμ (ref. 7).

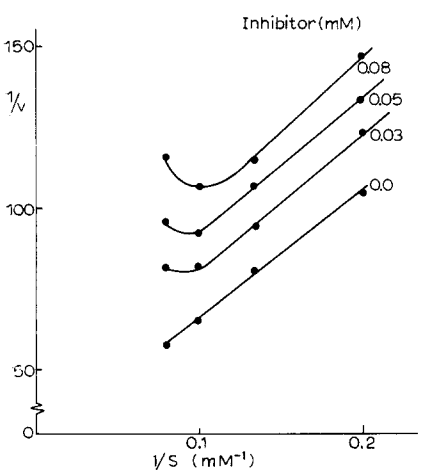

Fig. 3. Substrate inhibition type WEBB[15] plot obtained with 2,4-dichloronitrobenzene (0.50; 0.75; 1.0, and 1.25 mM) in the presence of sulphobromophthaleine (0, 0.03, 0.05 and 0.08 mM) regarded as an inhibitor. Reaction mixtures, containing glutathion (3.75 mM) and pigeon liver supernatant in 0.1 M pyrophosphate buffer (pH 8.0) were incubated at 37°. The enzyme activity, v, was measured by the increase in the absorbance at 344 mμ (ref. 7).

type of the nitrite ion release (see DISCUSSION), K_m values for TCNB and 4 nitropyridine *N*-oxide do not exist.

Effects of competitive substrates on glutathione conjugations

Sulphobromophthaleine is known to be a competitive inhibitor for rat liver enzyme with respect to the conjugation of DCNB–glutathione[4]. Working with pigeon liver supernatant, we found an "uncompetitive inhibition" type of plot (see ref. 14), as shown in Fig. 2. When plotted as $1/v$–$1/[S]$, substrate inhibition was observed with increasing concentrations of DCNB and sulphobromophthalein (Fig. 3).

With respect to nitrite ion releasing substrates, pigeon-liver supernatant is probably non-competitively inhibited by DCNB in its conjugation of glutathione with TCNB. The K_i value $4 \cdot 10^{-3}$ M is calculated from Fig. 4.

A similar experiment with rat liver supernatant gave irregular results and $4 \cdot 10^{-3}\ \text{M} < K_i < 8 \cdot 10^{-3}\ \text{M}$.

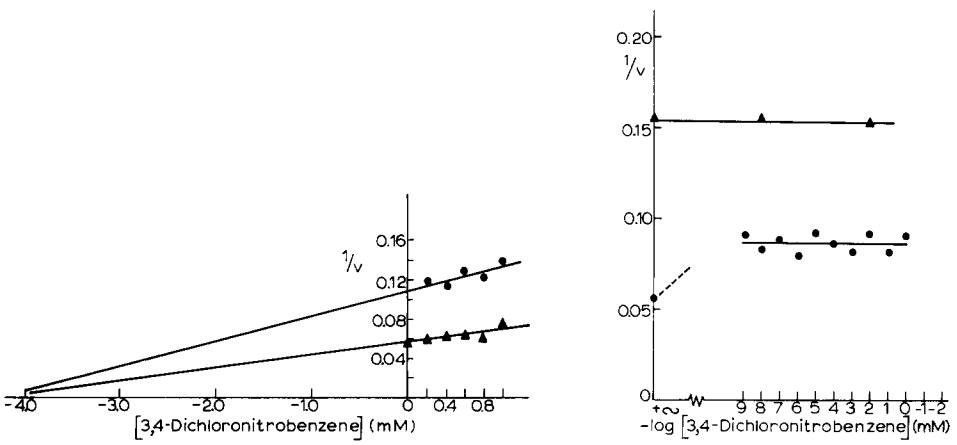

Fig. 4. Non-competitive DIXON[13] plot obtained by regarding 2,3,5,6-tetrachloronitrobenzene (●, 0.05 mM; ▲, 0.19 mM) as the substrate and 3,4-dichloronitrobenzene as the inhibitor. Incubation was for 15 min at 37° in the presence of 6 mM glutathione, and 0.1 M pyrophosphate buffer (pH 8.0). The enzyme activity was measured by the increase in nitrite release[12].

Fig. 5. DIXON[13] plot for 4-nitropyridine *N*-oxide (0.5 mM) as substrate and 3,4-dichloronitrobenzene as inhibitor. Incubation was for 15 min at 37° in the presence of 6 mM glutathione and 0.1 M pyrophosphate buffer (pH 8.0). The enzyme activity was measured by the increase in nitrite release[12]. ▲, release of nitrite in the incubation mixture without enzyme.

A qualitatively similar inhibition, *i.e.*, non-competitive, was expected as the effect of DCNB on the enzymic release of nitrite ions from 4-nitropyridine *N*-oxide. When the effect of DCNB on rat liver supernatant was examined, a K_i $2.4 \cdot 10^{-3}$ M was found which is comparable to the value recorded by AL KASSAB, BOYLAND AND WILLIAMS[12]. Pigeon liver supernatant gave a very remarkable result (Fig. 5), since the release of nitrite ions was inhibited to the same extent by DCNB over a large range of concentrations (10^{-9}–10 mM). An attempt to determine the formation of the glutathione conjugate with DCNB under these conditions failed, since the absorption curves of 4 nitropyridine *N*-oxide and *S*-(2-chloro, 4-nitrophenyl)-glutathione overlap to a large extent.

DISCUSSION

BRAY, JAMES AND THORPE[1] have summarized the metabolic fate of 19 chlorinated mononitrobenzenes in the rabbit. Many of these undergo mercapturic acid formation by replacement reactions. In addition these compounds are partly reduced and hydroxylated to give chlorinated anilines and aminophenols. When DCNB and TCNB are given orally to pigeons, DCNB is mainly metabolized by reduction, and mercapturic acid formation occurred only in traces, while TCNB forms only mercapturic acid, since there was no indication of the presence of aniline or aminophenol metabolites. The dose employed (1000 mg/kg body wt.) proved to be emetic.

Since the first step in mercapturic acid formation of DCNB and TCNB is enzymic conjugation with glutathione, it seemed probable that the degree of mercapturation is associated with the level of the conjugating enzymes (glutathione *S*-aryltransferases) in the liver. An examination of the enzyme activities *in vitro* supported this conclusion. The enzyme activity towards DCNB is low, while that towards TCNB is high. From the investigation of the enzymic glutathione conjugations with 2 additional substrates — 2,4-dinitrochlorobenzene (Cl replacement) and 4-nitropyridine *N*-oxide (NO_2 replacement) — it appeared that the nitro group substitution reactions occurred more readily than Cl replacement. In addition it appeared that there are differences in enzyme activity with respect to species and substrates for the same type of replacement (Table I). Rat and pigeon liver supernatants demonstrated close similarities but also gross differences. It was found that for DCNB, K_m is $1.0 \cdot 10^{-3}$ M for both species. For TCNB and 4-nitropyridine *N*-oxide, the release of nitrite ions is linear with increasing substrate concentrations[12]. For 2,4-dinitrochlorobenzene, the K_m value could not be determined because of the non-linearity of the $1/v$–$1/[S]$ curve for both rat and pigeon liver preparations (not shown).

Gross differences became apparent when rat liver and pigeon liver supernatants were examined by competition experiments. Sulphobromophthalein is a competitive inhibitor for the rat liver enzyme, but inhibits the pigeon liver enzyme uncompetitively, when the conjugation of DCNB with glutathione is considered (Fig. 2). A second species difference was obtained by using DCNB as inhibitor for the nitrite-ion-releasing substrates TCNB and 4-nitropyridine *N*-oxide. DCNB inhibits the conjugation of glutathione with TCNB non-competitively. Rat liver preparations are non-competitively inhibited by DCNB in releasing nitrite ions from 4-nitropyridine *N*-oxide[12], but under the same experimental conditions, DCNB inhibits the pigeon liver preparation over a very wide range of concentrations (Fig. 5). A plausible explanation cannot yet be advanced.

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