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Glutathione S-transferases liberate hydrogen cyanide from organic thiocyanates*

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ORGANIC thiocyanate insecticide chemicals are useful in control of household, veterinary, and agricultural pests. Those in current use for insect control include the following compounds: isobornyl thiocyanatoacetate (Thanite®), 2-(2-butoxyethoxy)ethyl thiocyanate (Lethane 384®), and 2-thiocyanatoethyl laurate (Lethane 60®).[1,2] Thanite, substituted-benzyl thiocyanates, and related compounds synergize the insecticidal activity of carbaryl, propoxur, and pyrethrins.[2–5] The thiocyanato group is sometimes introduced into candidate pesticide chemicals not only to serve as a possible toxophore but also to confer other desirable physicochemical properties to the molecule. Although the commercial thiocyanate insecticide chemicals have a low acute toxicity to mammals,[6–12] some of the lower alkyl analogs are highly toxic.[8, 13–15]

There is evidence that, in both insects and mammals, *in vivo* liberation of hydrogen cyanide is the cause of toxicity of some organic thiocyanates, particularly the lower aliphatic homologs.[8,9,13,14,16,17] Hydrogen cyanide is detectable in the blood of rabbits administered tetramethylene thiocyanohydrin and, as anticipated, *p*-aminopropiophenone protects against lethal doses of this thiocyanate by formation of methemoglobin; another indication of cyanide-like action is that tetramethylene thiocyanohydrin produces a rise of blood pressure in artificially respired cats.[13] Rabbit liver pulp forms hydrogen cyanide in large amount from methyl and ethyl thiocyanates and in small amount from butyl and 2-(2-butoxyethoxy)-ethyl thiocyanates during incubation for 48 hr at 37°,[14] but previous work does not reveal the nature of the enzyme(s) involved, if any.

The present study establishes the important role glutathione S-transferases play in the formation of hydrogen cyanide from a variety of organic thiocyanates by mouse liver and housefly homogenates.

Homogenates of mouse liver and whole houseflies (*Musca domestica* L.) prepared at 20% (w/v) in 0·1 M phosphate buffer, pH 7·4, were separated into the following centrifugal fractions: mitochondria and debris (sedimented at 20,000 *g* for 20 min and discarded); microsomes (sedimented at 105,000 *g* for 40 min but not at 20,000 *g* for 20 min); supernatant (not sedimented). Proteins in the supernatant fraction were chromatographically separated at 5° on two types of columns: Sephadex G-25 eluting with 0·1 M phosphate buffer, pH 7·4, to obtain the portion designated "soluble fraction"; Sephadex G-25 eluting with 0·005 M tris–HCl buffer, pH 7·0, followed immediately by DEAE cellulose eluting with tris–HCl buffer gradients, the latter according to Moore and Lee.[18]

The homogenate fractions were assayed for activity in degrading organic thiocyanates by incubating 1·5–4 mg of protein with substrate (500 mμmoles) and glutathione (GSH), (0 or 1000 mμmoles) in 1·0 ml 0·1 M phosphate buffer, pH 7·4, for 30 min at 37°. The amount of hydrogen cyanide liberated was determined colorimetrically according to Bruce *et al.*[19] Specific enzyme activity was expressed as mμmole hydrogen cyanide released/min/mg protein, with protein determination by the Folin–Ciocalteu method.[20] Reaction products other than hydrogen cyanide were fractionated, by extraction with ether, into ether- and water-soluble materials, and they were identified by thin-layer chromatography (silica gel G) and paper chromatography (Whatman No. 1), respectively. Ether-soluble products were separated by developing the chromatograms with hexane or hexane–ether mixture (100:1) and the sulfur compounds were detected with iodine. Glutathione and its derivatives in the aqueous phase were chromatographically separated with butanol–acetic acid–water mixture (4:1:2) or phenol–water mixture (4:1) and detected with ninhydrin.

Mouse liver preparations. An enzyme in the supernatant fraction of mouse liver homogenate rapidly forms hydrogen cyanide from heptyl or octyl thiocyanate but the supernatant fraction does not have this activity after it is heated at 80° for 10 min prior to its use. The activity of the microsomal fraction, with or without fortification with NADPH, is very low in comparison with the supernatant fraction. The hydrogen cyanide-producing activity of the supernatant fraction is greatly reduced on passage through a Sephadex G-25 column but addition of GSH to the recovered protein (i.e. the portion designated "soluble fraction") restores the activity to a level approximating that of the original supernatant fraction. Two of the four protein fractions separated from the supernatant preparation on DEAE cellulose are almost equally active, when fortified with GSH, forming hydrogen cyanide from heptyl thiocyanate. These same two fractions, eluting with 0·005 M and 0·1 M tris–HCl, pH 7·0, also contain almost all of the activity for degrading Sumithion® [*O,O*-dimethyl *O*-(4-nitro-*m*-tolyl)

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phosphorothionate] in the presence of GSH, indicating that the same enzyme(s) may be responsible for metabolism of Sumithion and alkyl thiocyanates. Sumithion cleavage involves methyl transfer from the dimethylphosphorothionate to GSH by glutathione S-transferase(s).[21,22] When assayed with methyl- or 2-(2-butoxyethoxy)ethyl thiocyanates, as substrates, not only the two protein fractions noted above are active but a third fraction, eluting with 1·0 M NaCl in 0·33 M tris–HCl, pH 8·0, also shows high activity. Thus, several glutathione S-transferase enzymes in mouse liver soluble fraction produce hydrogen cyanide from alkyl thiocyanates and this fact adds organic thiocyanates to the list of substrates already known to react with these enzymes.[23,24]

The optimum chain length for hydrogen cyanide formation from alkyl thiocyanates by mouse liver enzymes is found with heptyl or octyl thiocyanate (Fig. 1). Lethane 384 is a reactive substrate, rapidly yielding hydrogen cyanide, but Thanite is not. The thiocyanates containing the following substituents yield large amounts of hydrogen cyanide in the presence of GSH alone, although the rate of reaction

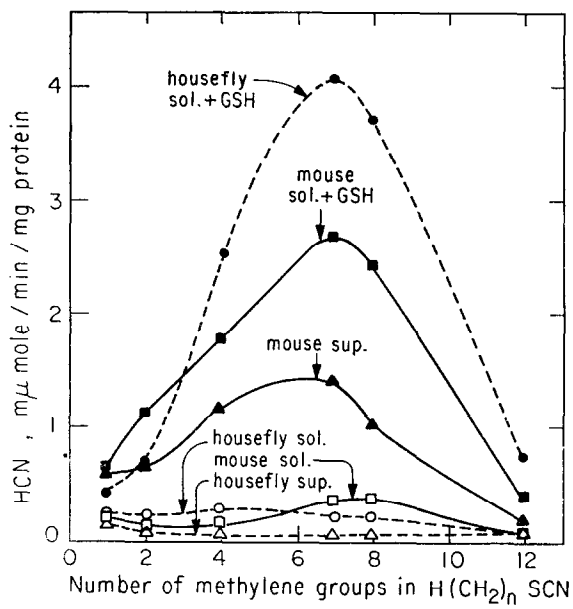


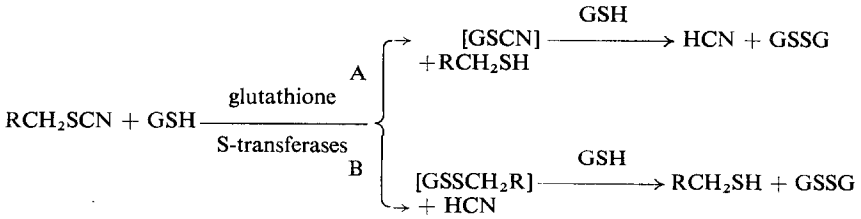
FIG. 1. Substrate-specificity of mouse liver- or housefly homogenate fractions for liberation of hydrogen cyanide form alkyl thiocyanate homologs.

increases in the presence of the enzyme systems: chloromethyl, benzyl, *p*-chlorobenzoylmethyl, 2-naphthoylmethyl, phenyl, substituted-phenyl methylcarbamate. The reaction of methyl- and ethyl thiocyanates with GSH, in the presence or absence of enzyme, yields, thiocyanide as well as hydrogen cyanide but ethyl isothiocyanate does not, under the reaction conditions used.

Housefly preparations. The supernatant from housefly homogenates contains little activity for forming hydrogen cyanide from organic thiocyanates either before or after passage through Sephadex G-25. The addition of GSH to these fractions, however, causes a marked stimulation in activity not evident with GSH alone. The substrate specificity of the homogenate fraction(s) with respect to the optimum chain length in a series of alkyl thiocyanates is similar to that of the mouse liver fraction(s) (Fig. 1). Glutathione may be the limiting factor for activity in housefly homogenates but not in mouse liver homogenates; in this respect, it is known that rat liver contains 5·5 times more GSH than the midgut of horn beetle larvae.[21]

Reaction mechanism. There are three identified products from the metabolism of organic thiocyanates by mouse liver enzyme preparations: hydrogen cyanide; oxidized glutathione (GSSG) present in each case in the aqueous phase; the mercaptan released on thiocyanate cleavage or the disulfide formed by oxidation of this mercaptan during isolation as the major ether-soluble products. Thus, octyl thiocyanate yields octyl mercaptan and benzyl thiocyanate yields dibenzyl disulfide. The identified products can be generated by either one of two mechanisms involving enzymatically-

mediated attack of the GSH at the cyano grouping (mechanism A) or at the thiocyanate sulfur (mechanism B), as follows:



It is not known which of these two mechanisms is involved.

Glutathione S-transferases frequently serve to detoxify xenobiotics by catalyzing an initial stage in mercapturic acid biosynthesis.[23] However, in the case of organic thiocyanates, liberation of hydrogen cyanide can be considered, in some cases, to be an activation process because it is likely that the liberated hydrogen cyanide accounts for some or all of the toxic effect from poisoning with some of these materials. With other organic thiocyanates containing an additional but different type of toxophoric grouping (such as the 4-thiocyanatophenyl methylcarbamates with high anticholinesterase activity but low insecticidal activity),[25] metabolic attack at the thiocyanato group, although liberating hydrogen cyanide, possibly destroys the compound before lethal action by the second toxophore is achieved. Thus, depending on the compound, organic thiocyanates undergo either activation or detoxification in the presence of glutathion S-transferases.

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Metabolism and anticonvulsant activity of diazepam in guinea pigs

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PREVIOUS studies have shown that diazepam, incubated *in vitro* with liver microsomes of different animal species[1,2] is hydroxylated and *N*-demethylated in rats and mice while it is only *N*-demethylated in guinea pigs.

In an effort to compare the metabolism of diazepam with the anticonvulsant activity of this benzodiazepine a study was made for investigating these aspects in guinea pigs according to a pattern previously employed in rats and in mice.[3]

Male Albino guinea pigs (body wt. 300–350 g) were used in all experiments.

Diazepam was administered by intravenous injection at a dose of 5 mg/kg dissolved in a solvent containing propylglycol–glycofurol–benzyl alcohol–water (30:30:2:48). Metrazol was injected i.p. at different doses and at different times after administration of diazepam, as specified below.

Extraction of diazepam and metabolites and their gas chromatographic determination. The preparation of blood, brain and adipose tissue extracts were made according to the method previously reported.[4] Gas chromatographic analyses were carried out using a gas chromatograph Model G_1 (Carlo Erba, Milan) equipped with a Ni 63 electron capture detector (Voltage 42 V).

The stationary phase was OV_1 3% on Gas Chrom Q (60–80 mesh) packed into a 4 m glass column (int. dia. 2 mm; ext. dia. 4 mm).

The flow rate of the carrier gas, nitrogen, was 33 ml/min, the column temperature was 245° and the injection temperature was 290°.

At various times after i.v. administration of diazepam, metrazol was injected intraperitoneally beginning with a dose of 100 mg/kg and increasing it by a factor of 1,2. Groups of six guinea pigs were observed for a period of 30 min after metrazol injection and their responses recorded. The parameter used for measuring antimetrazol activity was the relative number of animals undergoing clonic and tonic seizures.

The protection against the convulsant action of graded amounts of metrazol by pretreatment with diazepam injected i.v. at a fixed single dose of 5 mg/kg is shown in Fig. 1. Metrazol was given i.p. at different times after diazepam administration. Different doses were used beginning with 100 mg/kg (a 100 per cent lethal dose for guinea pigs) and increasing the dose by a factor of 1,2.

The anticonvulsant activity exerted by diazepam against metrazol declines with the time after diazepam administration, so that no protection against the convulsive response was observed when metrazol was given 10 hr after the pretreatment with diazepam.

In Table 1 is reported the metabolic pattern of diazepam in blood, brain and adipose tissue of guinea pigs. The highest blood and brain concentrations of intact diazepam were observed at 5 min after diazepam administration, but in the adipose tissue the concentration of intact diazepam reached a peak level only 30 min after diazepam administration.

Thereafter, in all tissues there was a gradual decline. The blood levels of diazepam were undetectable at 10 hr while in brain and in adipose tissue there were still measurable levels 20 hr after the intravenous administration of diazepam.