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THIAZOLIDINE DERIVATIVES AS SOURCE OF FREE L-CYSTEINE IN RAT TISSUE

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Abstract—The present study demonstrates that a variety of thiazolidine-4-(*R*)-carboxylic acids (TDs) which are the products of reactions of L-cysteine (cys) with carbonyl compounds could serve as a "delivery" system for cys to the cell. Liberation of the amino acid can occur enzymatically as well as non-enzymatically. The two possibilities have been proven by identification of representative compounds. The most specific substrate for mitochondrial enzymatic oxidation was thiazolidine-4-carboxylic acid (CF), the product of the reaction of cys with formaldehyde, and the least metabolized TD was 2-methyl-thiazolidine-4-carboxylic acid (CA), the product of the reaction of cys with acetaldehyde. TDs formed from cys and different sugars were not metabolized at all in mitochondria. *N*-Formyl-L-cysteine (NFC) the intermediate product of mitochondrial metabolism of CF was ascertained by ^{1}H-NMR spectroscopy whereas *N*-acetyl-L-cysteine (NAC), the predicted metabolite of CA, was not detected, possibly due to a fast turnover. The further enzymatic hydrolysis of NFC as well as NAC to free cys was demonstrated to take place in the cytoplasm. Non-enzymatic hydrolysis of TDs depended on the chemical nature of the substituents in the thiazolidine (Th) ring. The most stable compound was unsubstituted Th and the least stable were CGlu(D) and CA. Following non-enzymatic ring opening and hydrolysis, CA was converted to methyl-djenkolic acid, which equilibrates with CA. We have identified this new compound by ^{1}H-NMR spectroscopy. TDs may cause both a decrease and an increase in the levels of SH-groups in mitochondria. In the case of the stable CF, which is metabolized only enzymatically, an increase in the levels of SH-groups in mitochondria was observed. This suggests that enzymatic control of the breakdown of TDs prevents overflowing of the cell with thiol groups. The latter seems to be induced by high concentrations of those TDs which are hydrolysed non-enzymatically. This process leads finally to a decrease in free SH-groups by different mechanisms. The findings demonstrate two different mechanisms by which TDs can provide cys to the cells. The biological and pharmacological consequences are discussed.

It has been shown previously [1–3] that incubation of both tryptamine and 5-hydroxytryptamine with brain tissue results in the formation of the aldehydes followed by the thiazolidine-4(*R*)-carboxylic acids (TDs‖) and then by the respective indolic acids. The rationale of the new pathway of biogenic amine neurotransmitters might be the control of the chemically active aldehydes by rapid inactivation. The TDs are formed by a condensation reaction of L-cysteine (cys) not only with biogenic amine-derived aldehydes but also with a variety of other carbonyl compounds. For example, pyruvate, deriving either from cys or phosphoenol-pyruvate, condenses with cys yielding 2-methyl-thiazolidine-2,4-dicarboxylic acid (CP) (Structure 1) *in vitro* and *in vivo* [4–6]. Likewise retine [7], pyridoxal phosphate [8], formaldehyde [9, 10], acetaldehyde [11], acrolein [12], methylglyoxal [13] and desaturated aldehydes formed during peroxidation of lipids [14] can react non-enzymatically with cys to produce thiosemiacetals which, with close proximity of the amino group, closes the ring to form TD (Scheme 1). The many different substrates suggest that condensation with cys represents a general feature of intracellular carbonyl compound metabolism. Furthermore, the reaction regulates the level of cys in the cells. The necessity of such a mechanism becomes apparent with the evidence that cys is considered to be the most toxic amino acid [15] and that the physiological concentration is kept at very low levels, which protects against the toxicity of this amino acid [16].

Under normal conditions, the SH-groups of cys play a very significant role in the cell. They participate in the synthesis of proteins, are structural elements of proteins, are parts of the active centers of enzymes, and significantly effect mitosis [17, 18] and carcinogenesis [19]. However, marked decreases in

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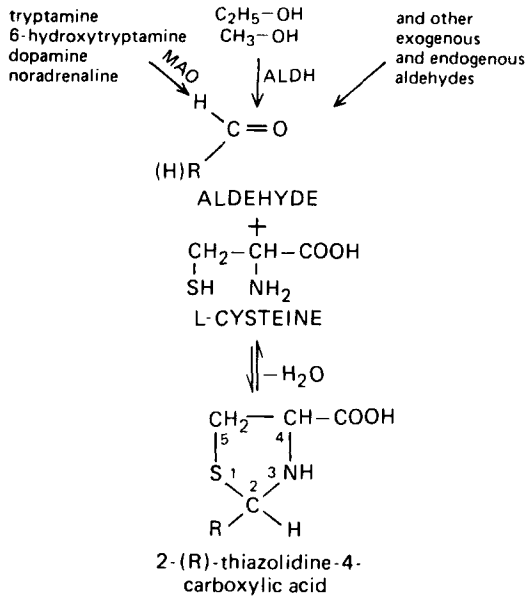
‖ Abbreviations: cys, L-cysteine; TD, thiazolidine-4(*R*)-carboxylic acid; TL, thiazoline derivatives; Th, thiazolidine; CF, thiazolidine-4(*R*)-carboxylic acid; CA, 2-methyl-thiazolidine-4(*R*)-carboxylic acid; CP, 2-methyl-thiazolidine-2,4-dicarboxylic acid; CIA, 4(*R*)-2-(3′-indolylmethyl)-1,3-thiazolidine-4-carboxylic acid; NFC, *N*-formyl-L-cysteine; NAC, *N*-acetyl-L-cysteine; GSH, reduced glutathione; DTNB, 3-carboxy-4-nitrophenyl disulfide (Ellman reagent); oABA, *ortho*-amino-benzaldehyde; MDA, methyl-djenkolic acid; DNFB, 2,4-dinitrofluorobenzene; TCA, trichloroacetic acid. The abbreviations of individual TDs are in Table 1.



Scheme 1. Formation of thiazolidines from aldehydes and cys.

the levels of cys and reduced glutathione (GSH, a tripeptide which serves as a storage and transportation form of cys [20]) was observed in various pathological states [21, 22]. Recently, depletion of intra- and extracellular thiol levels was discovered in HIV-infected patients [23–25]. Cys is the rate-limiting amino acid in GSH synthesis [26] which is a major cellular defense factor against chemically reactive molecules in the body [27]. Modulation of GSH levels may be useful in chemotherapy and in radiation therapy and it may help to protect cells against the toxic effects of drugs and other xenobiotic compounds [28, 29].

As reported by Anderson and Meister [30], L-2-oxothiazolidine-4-carboxylate is enzymatically converted to cys in many mouse tissues. The biological function of the TDs could be that they are active substrates in intracellular release of cys. Such a possible function of TDs underlines the importance of regulation of the breakdown of TDs.

Until now, the studies concerning TDs have dealt almost exclusively with thiazolidine-4(*R*)-carboxylic acid (CF) (see Structure 1), the product of the reaction between cys and formaldehyde [31, 32]. CF displays anti-oxidative [33, 34], hepatoprotective [35, 36] and carcinostatic [37, 38] properties and can replace cys in rat diet [39]. The first aim of the present paper was to investigate the fate of a variety of TDs (Structure 1) in rat liver and brain mitochondria. We examined a number of TDs to determine whether they could serve as a non-toxic "delivery" system for cys and to establish to what extent the breakdown of the various TDs involves enzymatic processes and non-enzymatic solvolysis.

Another aspect of the present study should be mentioned. There is evidence that acetaldehyde condenses with cys [10, 11]. Supposing that during ethanol intoxication the intracellular rise in acetaldehyde surpasses the capacity of aldehyde dehydrogenases; the formation of CA, its properties and further fate could then be of great importance.

MATERIALS AND METHODS

Animals. Female Wistar rats weighing 200–220 g were used in all experiments (Animal farm Hagemann Boesingfeld, F.R.G.).

Chemicals. 3-Carboxy-4-nitrophenyl disulfide (DTNB), GSH, *N*-acetyl-L-cysteine (NAC), and bovine serum albumin were obtained from Sigma Chemie (Deisenhofen, F.R.G.); L(+)cysteine, hexachloroplatin and materials for chromatography were purchased from Merck, Darmstadt (F.R.G.). *ortho*-Aminobenzaldehyde (oABA) was obtained from Fluka Chemie (Neu-Ulm, F.R.G.). DNFB (2,4-dinitrofluorobenzene) was from Aldrich (Steinheim, F.R.G.). *N*-Formyl-L-cysteine (NFC) was synthesized according to Mackenzie and Harris [32].

The following thiazolidine derivatives (Structure 1) were used: compound 1 = thiazolidine (Th) was obtained from Sigma; 2 = CF was purchased from EGA-Chemie (Steinheim, F.R.G.). 3 = CA was synthesized as described by Nagasawa *et al.* [11] and 4 = 2-methyl-thiazolidine-2,4-dicarboxylic acid (CP) according to Schubert [10] in the laboratory of organic synthesis, Jagellonian University, Krakow. 5 = 4(*R*)-2-(3′-indolylmethyl)-1,3-thiazolidine-4-carboxylic acid (CIA) was synthesized as described by Susilo *et al.* [2]. The syntheses of compounds 7–12 are described elsewhere [40] (method A). Compounds 6 and 13 were synthesized by a modified method of Weitzel *et al.* [41]. The stereochemistry of the products was determined by combined polarimetric and ^{1}H-NMR methods as described elsewhere [40].

The synthesis of methyl-djenkolic acid (MDA). MDA was synthesized according to Susilo [42]. Ten grams of cys was dissolved in 20 mL 6 M HCl and 2 g of acetaldehyde was added. After 2 hr of stirring, the reaction mixture was kept for 5 hr at room temperature and brought thereafter to pH 5.0 with 20% NaOH. After standing several hours at room temperature again, the product was collected by suction filtration, and washed with ethanol. The white solid phase obtained was two times recrystallized from a small volume of hot water after addition of several drops of 95% ethanol, to initiate precipitation of MDA (m.p. 240–244°).

Preparation of subcellular fractions and incubation procedures. The mitochondria and cytosol from liver and brain were prepared according to Gray and Whittaker [43]. Suspensions of mitochondrial fractions (0.25 mL) and 3 mM of TDs were incubated in 0.1 M phosphate buffer, pH 7.4, at 37° in a final volume of 0.5 mL. The reaction was stopped by addition of 0.5 mL acetonitrile after 15, 30 and 60 min. The tubes were chilled on ice and centrifuged (10,000 *g*, +4°, 15 min). The absorbance at 250 nm was determined and corrected for the absorbance of the control samples which had received just acetonitrile at zero time (0.5 mL before TD addition).

Determination of SH-groups in mitochondria as well as non-enzymatic hydrolysis (solvolysis) of TDs.

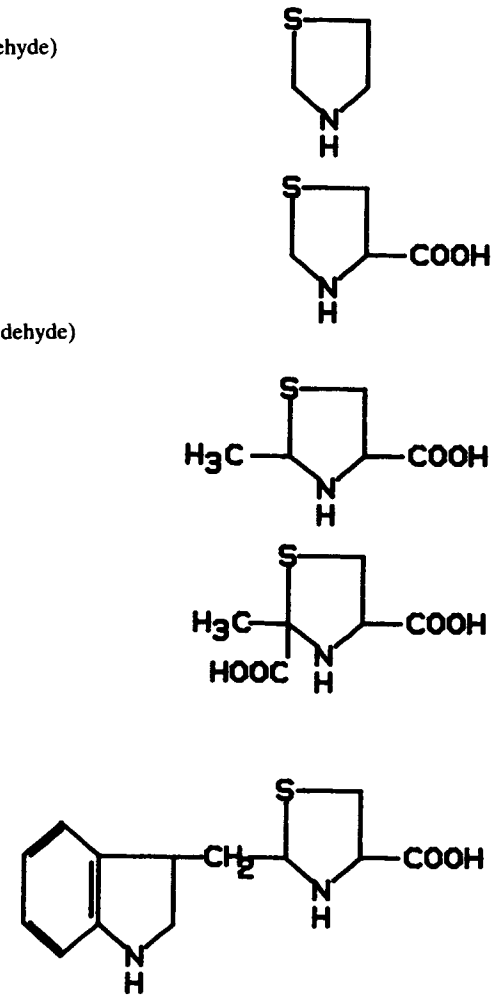
(1) Th (cysteamine + formaldehyde)

(2) CF (cys + formaldehyde)

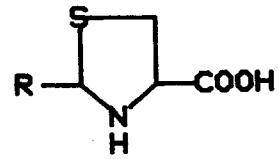
(3) CA (cys + acetaldehyde)

(4) CP (cys + pyruvate)

(5) CIA (cys + indole-3-acetaldehyde)



TDs, derived from sugars:



(6) R = 2(*S*)-D-gluco-pentahydroxypentyl, CGlu(D)
(7) R = 2(*S*)-D-arabino-tetrahydroxybutyl, CAra(D)
(8) R = 2(*R*)-L-arabino-tetrahydroxybutyl, CAra(L)
(9) R = 2(*R*)-D-fuco-tetrahydroxypentyl, CFuc(D)
(10) R = 2(*S*)-L-fuco-tetrahydroxypentyl, CFuc(L)
(11) R = 2(*R*)-D-galacto-pentahydroxypentyl, CGal(D)
(12) R = 2(*S*)-D-ribo-tetrahydroxybutyl, CRib(D)
(13) R = 2(*S*)-L-xylo-tetrahydroxybutyl, CXyl(L)

Structure 1. The 2-substituted TDs used in our experiments.

The amounts of SH-groups were assessed, using a modified method of Jocelyn [44]. The incubation mixture contained 2.5 mM TDs, 0.2 mL of mitochondrial suspension (1.2–1.4 mg of protein) in 0.1 M phosphate buffer, pH 7.4, in a final volume of 1 mL. After 30 min of incubation at 37°, 1 mL of 2 mM DTNB was added, the solution was stirred and the absorbance at 412 nm was determined 1 min later (photometer Zeiss PMQ). The results were corrected for hydrolysis which was determined in samples without mitochondria, e.g. non-enzymatic hydrolysis. The controls of Fig. 3 and Fig. 4 were performed without TDs (endogenous SH-groups levels).

Chromatography of metabolites of CF and CA. The metabolism of CF and CA was investigated in more detail. The products of CF and CA breakdown in the liver mitochondria were determined using TLC. CF or CA (5 mM) was incubated with 0.25 mL of mitochondrial suspension in 0.1 M phosphate buffer, pH 7.4, in a final volume of 0.5 mL. After 30 min, 0.5 mL acetonitrile was added to the incubation mixture which was then centrifuged (10,000 *g*, 4°, 30 min). Supernatant (10–20 μL) was analysed by TLC on silica gel 60 F_{254} plates using (for CF metabolites): solvent I, *n*-butanol/acetic acid/water (3:1:1), or solvent II, water saturated phenol; and for CA metabolites: solvent III methanol/methyl acetate (1:2).

The compounds were visualized using 0.2% ninhydrin reagent, 10 mM DTNB for thiol compounds [45], platinum reagent for sulfur compounds [46] and oABA (1% in acetone) for thiazoline derivatives (TLs; cyclic desaturated rings) [47].

Chromatography products of CA and CF conversion after derivatization with DNFB. Incubation procedure: suspensions of mitochondrial fraction (14 mL) were incubated at 37° in 0.75 mM phosphate buffer pH 7.4, with 42 mg of CA or 40 mg of CF, in a final volume of 28 mL. There were two control samples, one without mitochondria and the other without TDs. The reaction was stopped after 60 min by addition of Na_2CO_3 to reach pH 8.5–8.8 and 28 mL of acetone, and centrifuged for 10 min at 7800 *g*. The samples were then derivatized with 52 mg of DNFB per sample [46] and stirring for 60 min at room temperature, followed by addition of 6 M HCl to adjust the sample to pH 3.0. The organic phase was evaporated and the remainder was extracted three times with ethyl acetate which was finally evaporated to dryness. The residue was dissolved in a small volume of methanol and analysed to TLC silica gel, 60 F_{254}, 0.5 mM using $CHCl_2$, methanol 2:1 (solvent IV). The spots corresponding to MDA in the case of CA and NFC in the case of CF were scraped off and eluted with methanol, and evaporated to dryness. For preparation of samples for ^{1}H-NMR analysis the chromatography was repeated once more.

Effect of CF, CA and CGlu(D) on SH-groups of GSH and bovine serum albumin. Bovine serum albumin (2 mg/1 mL) was incubated for 30 min at 37° in 0.1 M phosphate buffer, pH 7.4, with TDs in a final volume of 1 mL. The concentrations ranged from 0.1 to 0.5 mM. Then 1 mL of 10 mM DTNB was added and after 60 min the absorbance was determined at 412 nm.

GSH (0.1 mM) was incubated for 30 min with TDs (0.1–0.5 mM concentration range) in 0.1 M phosphate buffer, pH 7.4, in 1 mL final volume. Thereafter, 1 mL of 2 mM DTNB solution was added and after 1 min, the absorbance was measured at 412 nm photometrically. The results were corrected by hydrolytic release of SH-groups from TD in samples without GSH and albumin. The samples without TD were used as control.

The determination of the K_m *and* V_{max} *of cys liberation from NAC and NFC in liver and brain cytosol.* The incubation mixtures for determination of initial velocities of NAC hydrolase contained in a final volume of 1 mL: NAC or NFC (0.5–50 mM), 0.5 mL of cytosol (16 mg protein/mL) in 0.1 M phosphate buffer, pH 7.5. The incubation was performed at 30° and stopped by addition of the same volume of 5% trichloroacetic acid (TCA) and centrifuged. The release of cys from NAC was determined in TCA supernatant according to Gaitonde [48] using ninhydrin reagent (250 mg ninhydrin in a mixture of 6 mL of acetic acid and 4 mL of conc. HCl). In the case of NFC, less stable in acidic solution, the liberated cys was determined in TCA supernatant with 2.5% ninhydrin ethanol solution. In both cases the tubes were covered with glass marbles and heated in boiling water for 5 min. They were then rapidly cooled and diluted with 1 mL of ethanol and the absorbance at 560 nm was measured. For calculations of K_m and maximal velocity the program of Lutz *et al.* was used [49].

No hydrolysis of NAC and NFC by liver and brain mitochondria was observed. The protein concentration was determined according to Lowry *et al.* [50].

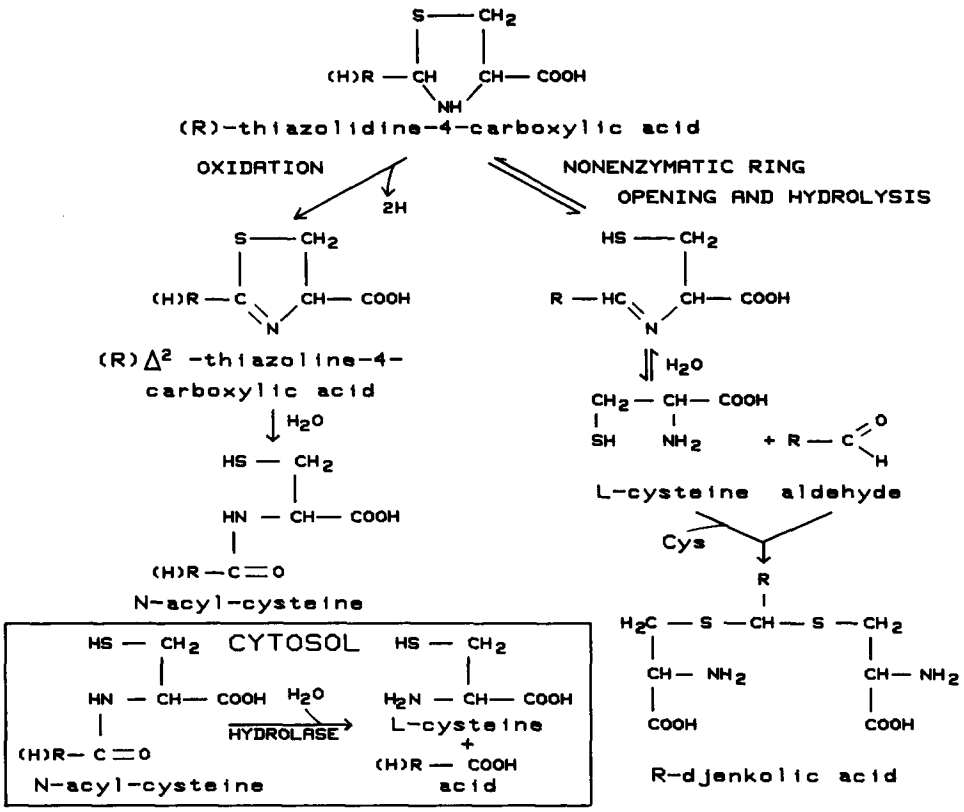
RESULTS

Oxidation of TDs to TLs

TDs which are fully saturated heterocyclics, do not show characteristic absorbance in UV light. The TLs however [51], having one double bond in the ring, show this feature and can be regarded as oxidation products of the former. Hence, by measuring absorbance in UV light following incubation of various TDs with subcellular fractions from rat brain and liver, it is possible to study the oxidation of the Th ring to the thiazoline ring. The results (Fig. 1) indicate that the increase in absorbance in UV light occurs with apparent substrate specificity and as a saturable process. Among the compounds studied, the most specific substrate for mitochondrial oxidase from the brain and liver (the maximum increase in absorbance at 250 nm) was compound CF. Correspondingly less active substrates were CP and CIA and the weakest CA. In liver mitochondria this reaction proceeded more intensively than in cerebral mitochondria. In contrast to the cerebral mitochondria, CP was oxidized more actively than CIA. Th and all TDs formed from sugars were not oxidized by either brain or liver mitochondrial fractions (Fig. 1).

Metabolism of TDs

CF was chosen as the prototype compound to



Scheme 2. Breakdown of TDs by an enzymatic (left) and a non-enzymatic pathway (right).

investigate the fate of thiazolidine carboxylic acids in liver mitochondria (Fig. 1). TLC in solvent I and solvent II followed by staining with ninhydrin, platinic iodine and DTNB revealed NFC (R_f 0.6 solvent I, 0.28 solvent II), *N,N'* diformyl-cystine (R_f 0.26 solvent I, 0.11 solvent II), confirming previous observations of Mackenzie and Harris [32], and CF (R_f 0.34 solvent I, 0.38 solvent II). In addition, an unidentified sulfur compound with $R_f = 0.18$ in solvent I and $R_f = 0.15$ in solvent II was detected. Although spectrophotometric studies suggested the occurrence of TLs as intermediate metabolites of CF in mitochondria (Fig. 1 and Scheme 2) the thiazoline could not be demonstrated by chromatographic methods. After staining with oABA (a reagent characteristic for TLs [47]), no typical colour reaction could be detected.

The study was extended to CA to investigate whether such degradation reactions occur in mitochondria with other TDs as well. In the case of CA (TLC in solvent III), not even traces of NAC but cys and its oxidation product cystine were identified.

Identification of NFC as a product of the enzymatic oxidation of CF in liver mitochondria after derivatization with DNFB

TLC in solvent IV proved NFC ($R_f = 0.29$) to be a product of the mitochondrial oxidation of CF. The identity of NFC was ascertained by ^{1}H-NMR after transformation into the *S*-(2',4'-dinitrophenyl)-derivative and purification by preparative TLC. The isolated and the authentic sample clearly show identical signals (Table 1). Thiazoline-4-carboxylic acid as a product of the first step of oxidation of CF (Scheme 2) was not detected.

Identification of MDA as a product of non-enzymatic conversion of CA at physiological pH

TLC in solvent IV revealed MDA as a product of CA conversion ($R_f = 0.175$) in samples with or without mitochondria (control). In addition, three other unidentified compounds were detected. The identity of MDA was ascertained by H^1-NMR comparison of the *N,N'*-bis-(2',4'-dinitrophenyl) derivative with an authentic sample (Table 2). Unexpectantly, most signals in both samples show doubling with equal intensity. ^{1}H-NMR clearly shows the same signals of the sample as of the authentic substance. In the case of CA after derivatization with DNFB, NAC was also not detected in samples with mitochondria.

Kinetics of cys liberation from NAC and NFC in liver and brain cytosol

The biodegradation of NAC and NFC takes place only in the cytosolic fraction and not in mitochondria. Table 3 shows the K_m and V_{max} of cys formation from NAC and NFC. Cys liberated in the cytosol may be further metabolized; nevertheless, the

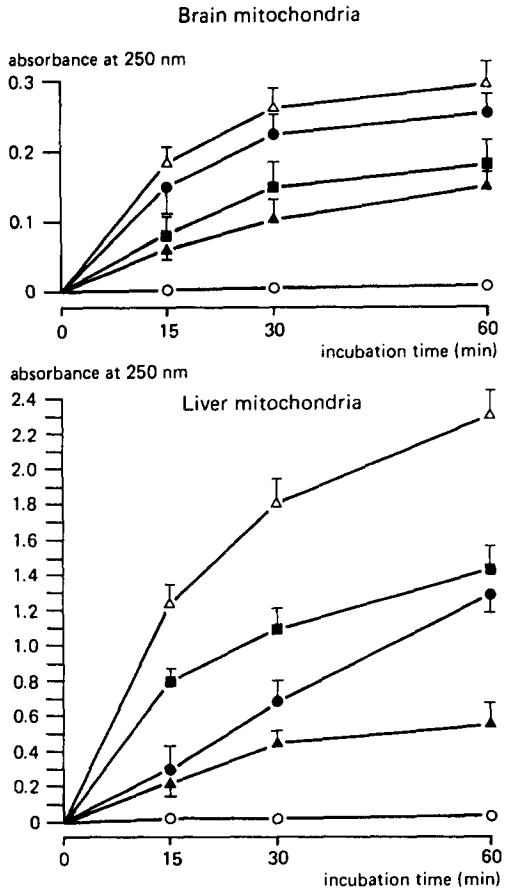


Fig. 1. Time course of the metabolism of TDs. Incubation mixtures contained 3 mM TD, 0.25 mL suspension of mitochondrial fraction (6–7 mg protein/1 mL) in 0.1 M phosphate buffer, pH 7.4, in a final volume of 0.5 mL. Compounds under study: CF (△); CP (■); CIA (●); CA (▲); Th and cys + sugars (○). Values are the means and SD from 5 to 10 experiments.

Table 1. ^{1}H-NMR data of *N*-formyl-*S*-(2′,4′-dinitrophenyl)-cysteine in CD_3OD at 300 MHz

Proton	(ppm)	Multiplicity	*J*(Hz)
2-H	4.69	ddd	8.1; 4.6; 0.4
3-H_a	3.54	dd	13.5; 8.1
3-H_b	3.78	dd	13.5; 4.6
3′-H	9.02	d	1.3
5′-H	8.49	dd	8.6; 1.3
6′-H	8.07	d	8.6
Formyl	8.15	d	0.4

enzymatic data from these experiments allow comparison of the specificity of an amino acid *N*-deacylase for the two substrates, and the specificity toward NFC in liver and brain cytosol.

Table 2. ^{1}H-NMR of the *N,N*′-bis-(2′,4′-dinitrophenyl) derivative of methyl-djenkolic acid in $CDCl_3/CD_3OD$ (1:1) at 300 MHz

Proton	(ppm)	Multiplicity	*J*(Hz)
2-H	4.66; 4.69	t	5.6; 5.3 Hz
3-H_a	3.24; 3.30	dd	13.5; 5.0; 13.8; 6.0
3-H_b	3.35; 3.39	dd	13.5; 5.5; 13.8; 5.0
4-H	4.12; 4.13	q	7.0; 7.0
5-H_3	1.60	d	7.0
3′-H	9.07; 9.08	d	2.9
5′-H	8.28; 8.30	dd	9.5; 2.9
6′-H	6.98; 6.99	d	9.5
N-H*	9.60; 9.65	d(br)	5

* Observed only in D_6-DMSO.

Table 3. Kinetic values of cytosolic amino acid *N*-deacylase

Substrate	Apparent K_m (mol/L)	Maximal velocity V_{max} (μmol/mL × min × mg protein)
Liver cytosol		
NAC	6.5×10^{-4}	0.016
NFC	1.7×10^{-3}	0.062
Brain cytosol		
NFC	2.4×10^{-3}	0.160

Non-enzymatic hydrolysis of TDs

Non-enzymatic opening of the Th ring (solvolysis, Scheme 2) was determined by measuring the concentration of cys SH- groups released from TDs during 30 min of incubation at 37° in 0.1 M phosphate buffer, pH 7.4 (Fig. 2). It turned out that the intensity of solvolysis varied and depended on the chemical properties of substituents in the Th ring. The least stable compounds were CGlu(D) and CA, while the heterocyclic unsubstituted Th ring was the most stable.

Effect of TDs on the concentration of SH-groups in tissue

Biological and possibly pharmacological effects of TDs depend to a great extent on the effects exerted upon SH-groups in the cell. We found that TDs may cause both a decrease and an increase in the level of SH-groups in mitochondria dependent on the compound (Fig. 3) and its concentration. The TDs with the chemically least stable ring, namely CA and CGlu(D) (see Fig. 2), led to a decrease in SH-groups during incubation with mitochondria, compared with controls (Fig. 3). It is noteworthy that the effect of CA on the SH-groups of brain mitochondria was significantly more pronounced than on those of liver mitochondria. The control value represents the concentration of SH groups in mitochondria without exogenous TDs. CF, a compound undergoing enzymatic degradation in mitochondria (Fig. 1), led to an increase in SH-group levels in this subcellular

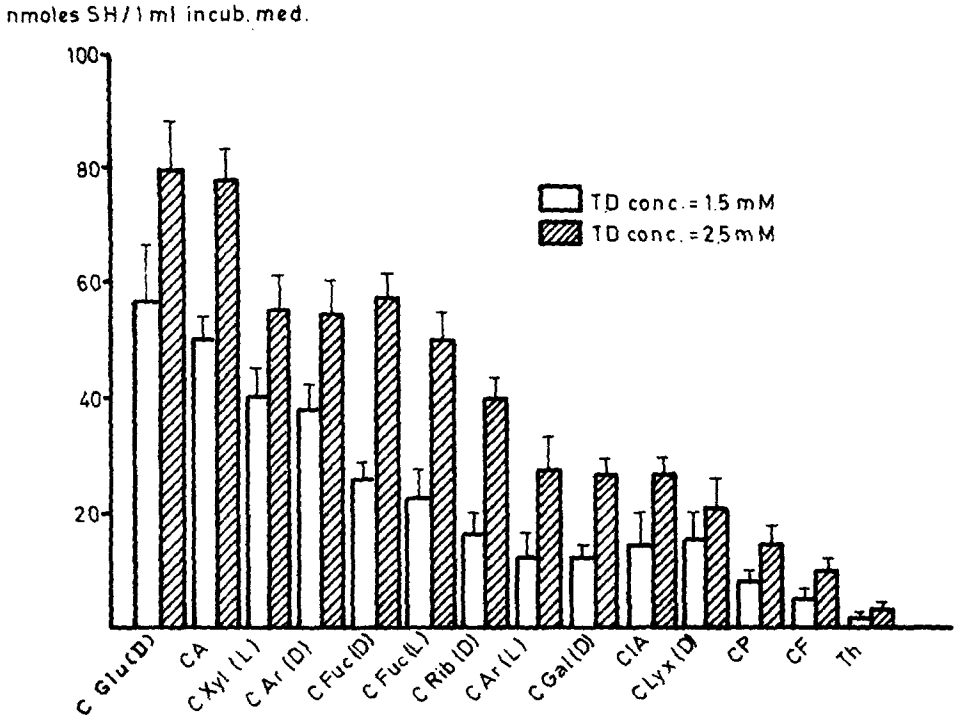


Fig. 2. Non-enzymatic hydrolysis of TDs. TDs at concentrations of 1.5 and 2.5 mM were incubated for 30 min at 37° in 0.1 M phosphate buffer, pH 7.4, in a final volume of 1 mL. The liberation of SH-groups of cys was estimated with DTNB. Values are means and SD of 5–10 experiments.

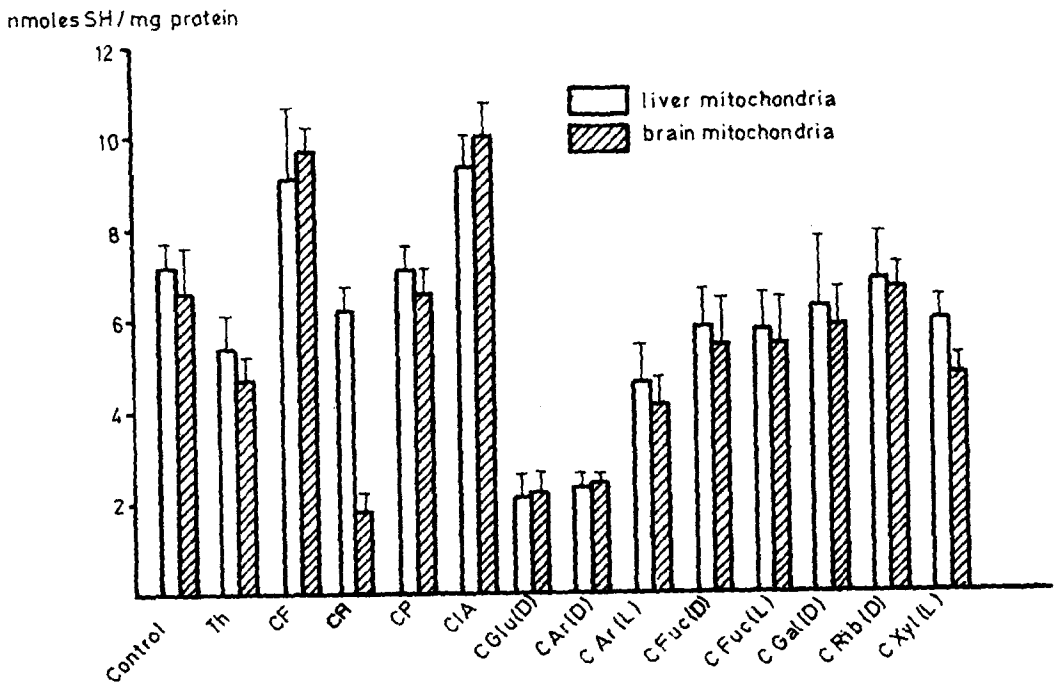


Fig. 3. The effect of TDs on the levels of low molecular SH-groups in liver and brain mitochondria. Mitochondrial suspension (0.3 mL, 1.2–1.4 mg protein) was incubated for 30 min with 2.5 mM TD in 0.1 MM phosphate buffer, pH 7.4, in a final volume of 1 mL. The SH-groups were estimated with DTNB. The results were corrected for non-enzymatic hydrolysis of TD. Samples incubated without TD were used as controls. Values shown are means and SD from 5 to 10 experiments.

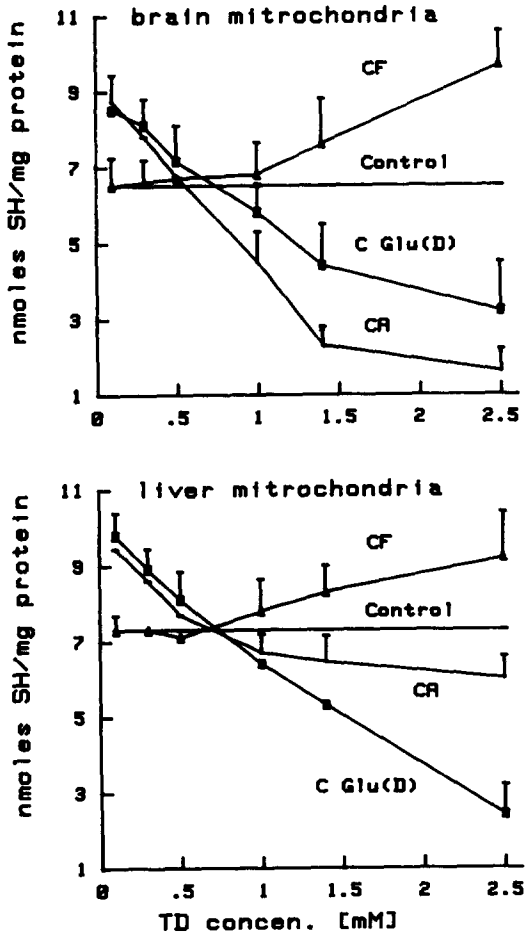


Fig. 4. The effect of different concentrations of CF, CGlu(D) and CA on free low molecular SH-groups in liver and brain mitochondria. (for details see legend to Fig. 3). Samples incubated without TD were used as controls. Values are means and SD of five experiments.

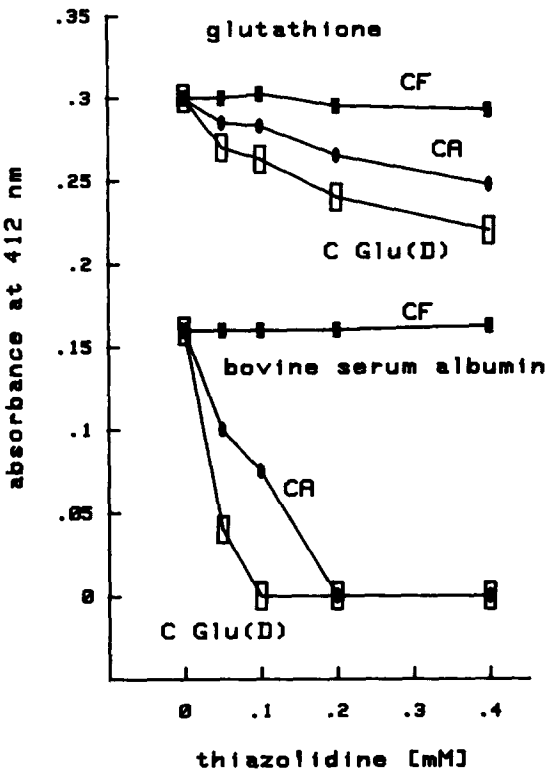


Fig. 5. The effect of different concentrations of CF, CGlu(D) and CA on SH-groups of GSH and bovine serum albumin. Bovine serum albumin (2 mg/1 mL) and 0.1 mM GSH were incubated in 0.1 mM phosphate buffer, pH 7.4, with TD in a final volume of 1 mL. The results were corrected for non-enzymatic hydrolysis of TD. Values are means and SD of five experiments.

fraction. To understand the basic mechanisms underlying the changes in SH-group levels, a study utilizing greater concentration ranges of CF, CA and CGlu(D) was performed with brain and liver mitochondria. The most remarkable findings were that low concentrations of CA and CGlu(D) (unstable compounds) induced an increase in SH-groups (Fig. 4). At approximately 0.6–0.8 mM no changes were observed and higher concentrations caused a reduction. CF behaved in a different way, presumably as a result of enzymatic degradation of CF followed by the formation of NFC.

CA and CGlu(D) induced a decrease in SH-groups in solutions of glutathione and bovine serum albumin (Fig. 5). This effect on free cys and in the case of CA also acetaldehyde must be caused by non-enzymatic hydrolysis while the stable CF did not reduce at all the amount of SH-groups of the two compounds (GSH and albumin).

In light of the present results, particular attention should be focused on the unsubstituted Th. Th incubated with mitochondria did not show an increase in absorbance in UV light (Fig. 1) which excludes oxidation to thiazoline. Moreover, it is a compound which does not undergo non-enzymatic hydrolysis (Fig. 2); nevertheless, Th caused a pronounced decrease in SH-groups in mitochondria (Fig. 3).

DISCUSSION

Cys plays a crucial role in essential cellular events as described in the introduction. In various pathological states marked decreases in the levels of cys and GSH have been reported [21–25]. Free cys cannot be used for therapeutic and experimental purposes because of its toxicity [15] and the ease of oxidation. We examined a number of TDs to determine whether they could serve as "frozen" cys. The basic notion of the present work was to investigate to what extent the breakdown of the various TDs to free cys involves enzymatic processes and non-enzymatic hydrolysis.

Spectrophotometric studies showed that some of the TDs under study were oxidized enzymatically to TLs, $(R)\Delta^2$-thiazoline-4-carboxylic acids, which are compounds with one double bond in the ring [40].

In the case of CP only the Δ^3-thiazoline derivative is possible. The most suitable substrate of mitochondrial oxidase was CF (Fig. 1). Several other compounds underwent enzymatic degradation to a variable degree. Other TDs, e.g. the carbohydrate derivatives and Th, are not oxidized at all by the mitochondrial enzyme. The further transformation of CF would proceed to NFC (Scheme 2) from which free cys is liberated by the action of an amino acid *N*-deacylase in the cytosol (Table 3). In conclusion, the breakdown of CF yields the acid and thus the re-emergence of free cys. As far as TLs are concerned, their occurrence has been supposed earlier by Cavallini *et al.* [31] and Mackenzie and Harris [32]. However, we have not detected measurable amounts of this compound by either HPLC or TLC, or by ^{1}H-NMR spectroscopy. So far, TLs have been identified as products of TD oxidation only under conditions where the reactions were catalysed by pure D-amino acid oxidases [52, 53]. The presence of one double bond in the ring makes them quite vulnerable to all types of nucleophilous attacks of SH, NH_2 and OH groups [54].

According to the results of our study, the capacity of the oxidase for CA in both liver and brain mitochondria is much less than for CF (Fig. 1). During incubation with mitochondria, only a slight net increase in absorbance at 250 nm was measured. Mackenzie and Harris [32] observed also a certain amount of oxygen consumption, which suggests oxidation of the Th ring. The lack of NAC in our experiments indicates that NAC occurs either in very low concentrations or is further converted to thioethers [55]. NAC can be further hydrolysed in the cytoplasm (Table 3). The low stability of CA that was observed by us (Fig. 2) is certainly the cause of not detecting this compound in the urine of rats given cys simultaneously with ethanol, or after administration of synthetic CA [56]. These observations do not, however, preclude the possibility that this compound could be produced *in vivo*. It has been found that cys may have a detoxication effect in acetaldehyde poisoning [57]. But it is not known if this effect results from a reaction with acetaldehyde (responsible for toxicity of ethanol), or from the activation of antioxidation systems by cys preventing peroxidation of lipids induced by ethanol and acetaldehyde [58].

It should be noted that those TDs which easily undergo solvolysis also introduce aldehydes to the cell. For example, in the case of CA, acetaldehyde is particularly toxic for the brain [59]. The liberated acetaldehyde can react with other SH-groups and new more stable derivatives may be formed. This contributes to the considerable fall in SH-groups of brain mitochondria following incubation with CA (Fig. 5). The smaller decrease in the liver mitochondria may be explained by the higher capacity of the acetaldehyde-metabolizing enzyme. The products liberated from TDs such as aldehydes and pyruvate can be metabolized by mitochondria thereby shifting the equilibria to the right.

Following the non-enzymatic ring opening of CA, MDA is formed, which was identified by ^{1}H-NMR (Table 2). The non-enzymatic reaction of cys with acetaldehyde to form MDA may be another method of acetaldehyde detoxification as an alternative to CA synthesis. MDA was demonstrated in hydrolysates of mammalian tissues [60]. It is not known if this compound serves in the presence of suitable C-S lyases as a "delivery" system for cys. It should be noted from Scheme 2 and the experimental data of this study that CA can be interconverted to MDA non-enzymatically at physiological pH. The equilibrium depends on pH as found in preliminary experiments: the lower the pH the more MDA is formed from CA.

Nagasawa *et al.* [61] have shown that CA administered at the same dose level as CF is less toxic and also provides effective protection against the toxicity of acetaminophen. CF is so far the only Th compound used as a hepatoprotective [35, 36] and anti-carcinogenic drug [37, 38]. But the therapeutic effects of this compound are associated with acute neurological symptoms observed mainly in children [62, 63]. Garnier *et al.* [63] suggest that the products of biodegradation of CF are responsible for the symptoms. Perhaps the products of further oxidation of NFC, i.e. *N*-formylcysteinesulfinic acid and *N*-formylcysteic acid can be blamed for CF neurotoxic effects [64, 65]. However, such a relationship has not been proven. The observed mechanism (Table 3) of enzymatic liberation of free cys from NFC in brain cytosol suggests that the toxicity of the CF is related to free cys in brain.

Free cys is well known to undergo also rapid spontaneous oxidation at neutral pH to form cystine, hydrogen peroxide and other free radicals [66] what also has deleterious effects on the free SH-groups in mitochondria. However, when enzymatic transformation is involved, e.g. in the case of CF in mitochondria, it is accompanied by an increase in SH-group levels. In this case, however, the increase reflects the formation of NFC, a thiol compound which we found to be the final product of CF metabolism in mitochondria (Table 1).

In this study, we have investigated the effect of TDs on non-protein SH-groups in mitochondria of the brain and liver (Fig. 3). We showed that in the case of labile, easily hydrolysed compounds the effects are variable and depend on concentration (Figs 3 and 4), e.g. CA and CGlu(D) at low concentrations (up to 0.4 mM) can protect the SH-groups but higher concentrations decrease the levels of SH-groups. A similar effect is exerted on SH-groups of GSH and bovine serum albumin (Fig. 5) which allows the conclusion that it is an effect of non-enzymatic hydrolysis and formation of mixed disulfides between endogenous mitochondrial SH-groups and liberated cys. In the case of CA reaction of acetaldehyde with SH-groups is also possible.

Among the compounds studied, CIA increases the level of SH-groups and brings about an increase in absorbance in UV light in mitochondrial fractions of both the liver and brain. This supports our earlier findings [3], in which CIA metabolism to indolo-3-acetic acid had been observed. All this indicates that CIA cannot be regarded only as a product of detoxification of indole-3-acetaldehyde. More research is necessary to explore the possibility of using CIA as "masked" cys particularly in brain cells

which are very sensitive to the toxicity of free cys [67, 68].

The most difficult problem to explain is the effect of the unsubstituted Th ring. This compound does not undergo either solvolysis (Fig. 2) or enzymatic oxidation (Fig. 2). However, it causes a decrease in the concentration of SH-groups in mitochondria from the liver and brain (Fig. 3). It has been shown that Th has clastogenic properties and suppress mitotic activity [69]. In order to inhibit mitosis in cancerous cells agents are searched for which block SH-groups, essential factors for cell division [13]. It is thus conceivable that the ability of Th to reduce SH-groups, observed in our experiments, could explain its antimitotic effect.

TDs may serve as the substrate for complex binding of metal cations [70] and also suppress carcinogenesis by blocking production of oncogenic *N*-nitroso compounds [71]. Furthermore, as reported by Roberts and Francetic [72], the product of condensation of cys with D-ribose selectively elevated GSH levels in numerous organs of tumor bearing mice.

In conclusion, TDs may effect essential processes in cells. The consequences can be beneficial but also harmful, depending on dose, stability, the chemical properties of an aminothiol and carbonyl compound from which TDs are formed, the specificity of mitochondrial oxidases and the biological properties of intermediates and final metabolites.

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