of P. Searle, J. S. Johansen, and S. Stilling, and the secretarial assistance of B. Hare are gratefully acknowledged. Dr. J. C. Watkins kindly supplied 2APV. Part of this work was performed at the Department of Medicinal Chemistry, University of Kansas, Lawrence, KS, and Drs. Mertes and L. A. Mitscher are acknowledged for fruitful discussions.

Registry No. 1, 82988-61-2; 2, 89017-61-8; 3, 89017-62-9; 4, 89017-63-0; 5, 65202-90-6; 6, 89017-64-1; 7, 89017-65-2; 8, 89017-66-3; 9, 65202-91-7; 10, 89017-67-4; 11, 89017-68-5; 12, 89017-69-6; 13, 75989-23-0; 14, 89017-70-9; 15, 89017-71-0; 16, 2417-73-4; 17, 89017-72-1; 18, 89017-73-2; 19, 89017-74-3; diethyl acetamidomalonate, 1068-90-2; 3-methoxy-8-(bromomethyl)-5,6-dihydro-4*H*-cyclohept[1,2-*d*]isoxazole, 89017-75-4; 3-methoxy-4-(bromomethyl)-7,8-dihydro-6*H*-cyclohept[1,2-*d*]isoxazole, 89017-76-5; (±)-ibotenic acid, 31758-99-3.

2-Substituted Thiazolidine-4(*R*)-carboxylic Acids as Prodrugs of L-Cysteine. Protection of Mice against Acetaminophen Hepatotoxicity

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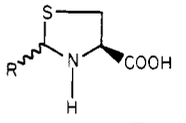
A number of 2-alkyl- and 2-aryl-substituted thiazolidine-4(*R*)-carboxylic acids were evaluated for their protective effect against hepatotoxic deaths produced in mice by LD_{90} doses of acetaminophen. 2(*RS*)-Methyl-, 2(*RS*)-*n*-propyl-, and 2(*RS*)-*n*-pentylthiazolidine-4(*R*)-carboxylic acids (compounds **1b**,**d**,**e**, respectively) were nearly equipotent in their protective effect based on the number of surviving animals at 48 h as well as by histological criteria. 2(*RS*)-Ethyl-, 2(*RS*)-phenyl-, and 2(*RS*)-(4-pyridyl)thiazolidine-4(*R*)-carboxylic acids (compounds **1c**,**f**,**g**) were less protective. The enantiomer of **1b**, viz., 2(*RS*)-methylthiazolidine-4(*S*)-carboxylic acid (**2b**), was totally ineffective in this regard. Thiazolidine-4(*R*)-carboxylic acid (**1a**), but not its enantiomer, **2a**, was a good substrate for a solubilized preparation of rat liver mitochondrial proline oxidase [$K_m = 1.1 \times 10^{-4}$ M; $V_{max} = 5.4$ μmol min^{-1} (mg of protein)$^{-1}$]. Compound **1b** was not a substrate for proline oxidase but dissociated to L-cysteine in this system. At physiological pH and temperature, the hydrogens on the methyl group of **1b** underwent deuterium exchange with solvent D_2O ($k_1 = 2.5 \times 10^{-5}$ s), suggesting that opening of the thiazolidine ring must have taken place. Indeed, **1b** labeled with ^{14}C in the 2 and methyl positions was rapidly metabolized by the rat to produce $^{14}CO_2$, 80% of the dose being excreted in this form in the expired air after 24 h. It is suggested that these 2-substituted thiazolidine-4(*R*)-carboxylic acids are *prodrugs of* L-*cysteine* that liberate this sulfhydryl amino acid in vivo by nonenzymatic ring opening, followed by solvolysis.

In an earlier communication,[1] we advanced the hypothesis that thiazolidine-4-carboxylic acids, in particular, the 2-substituted thiazolidine-4-carboxylic acids exemplified by 2(*RS*)-methylthiazolidine-4(*R*)-carboxylic acid (**1b**), can be considered to be prodrug forms of L-cysteine



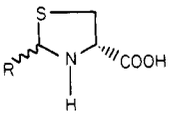
1a, R = H
b, R = CH_3
c, R = C_2H_5
d, R = *n*-C_3H_7
1e, R = *n*-C_5H_{11}
f, R = C_6H_4
g, R = 4-C_5H_4N

and provided evidence that **1b** can protect mice against acetaminophen-induced hepatic necrosis and death. In contrast, the parent thiazolidine-4(*R*)-carboxylic acid (**1a**) without alkyl substitution at C-2 was shown to be much less protective than **1b**; indeed, **1a** exhibited CNS toxicity at one-third the molar dose of **1b**.[1]

We now present evidence that other 2-alkyl- and 2-aryl-substituted thiazolidine-4(*R*)-carboxylic acids prepared from L-cysteine and the corresponding aldehydes are equally or nearly equally as protective as **1b** against acetaminophen-induced toxic deaths. We also show that (a) **1b** is not a substrate for rat liver mitochondrial proline oxidase; (b) the methyl group of **1b** undergoes deuterium exchange at physiological pH and temperature, a result possible only through opening of the thiazolidine ring; and (c) **1b** labeled with ^{14}C at the 2 and the methyl positions gives rise to the excretion of $^{14}CO_2$ in the expired air of mice administered the labeled compound, thereby supporting our premise that L-cysteine and acetaldehyde are liberated nonenzymatically in vivo from **1b**.

Results

Protection against Acetaminophen Toxicity by 2-Substituted Thiazolidine-4(*R*)-carboxylic Acids. The results shown in Table I suggest that, in general, 2-alkyl- and 2-phenylthiazolidine-4(*R*)-carboxylic acids (**1b** through **1f**) can protect mice against lethality by LD_{90} doses of acetaminophen even at half the molar dose of the latter. The 2-(4-pyridyl) analogue (**1g**) was not particularly effective in this regard, although no deaths were observed with this compound administered alone. The 2-ethyl analogue (**1c**) was less protective than **1b** but was comparable to the 2-phenyl analogue **1f** based on toxic deaths. However, the latter was much more protective than **1c** by histological criteria (Table I). Of mechanistic significance, whereas **1b** was protective, the enantiomeric 2(*RS*)-methylthiazolidine-4(*S*)-carboxylic acid (**2b**) derived from D-cysteine and acetaldehyde did not afford any protection.



2a, R = H
b, R = CH_3

† VA Medical Center and University of Minnesota.
‡ Hennepin County Medical Center.

(1) Nagasawa, H. T.; Goon, D. J. W.; Zera, R. T.; Yuzon, D. L. *J. Med. Chem.* **1979**, *25*, 489. Compound **1b** was called by its acronym, MTCA, here.

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Table I. Protection against Acetaminophen Toxicity in Mice by 2-Substituted Thiazolidine-4-carboxylic Acids

no.	survival (48 h):[a] acetaminophen[b] + test compd[c]	no. of animals with liver necrosis[d]				
		4+	3+	2+	1+	0
1b	28/30	1				17[e]
1c	19/24	6	3	3	3	3
1d	30/30	2		1	6	9
1e	24/24				2	16
1f	23/26	2	6	3		7
1g	13/24	6	5	1	4	2
2b	0/18	17	1			
10	17/17				2	15[e]

[a] Represents the cumulative results of at least three replicate experiments conducted at different times. [b] 750 mg (4.97 mol)/kg, ip. With acetaminophen alone, the cumulative survival rate from more than 10 experiments was 18/117 or 85% lethality at this dose regimen. [c] 2.45 mmol/kg, ip. No deaths were observed in controls where the test compounds were administered alone using 17–26 animals per test compound. [d] Due to high costs, histological sections were prepared from the first 18 animals only per test compound. The slides were coded and evaluated according to Mitchell et al.[31] The evaluator (R.T.Z.) did not have access to the keys of the code. [e] Data from an earlier communication (ref 1).

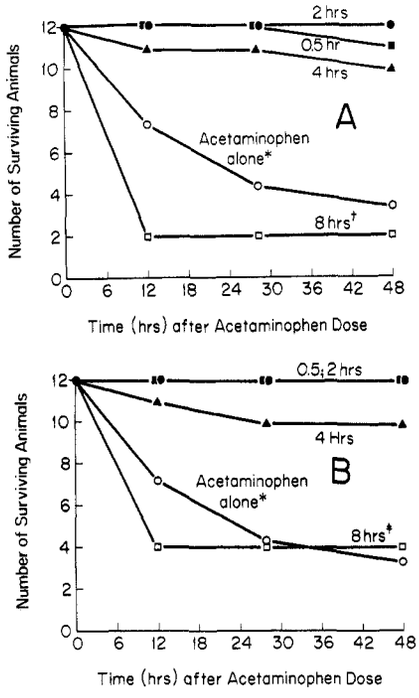


Figure 1. Effect of postadministration time of **1b** (A) or **1d** (B) on acetaminophen lethality in mice. Compound **1b** (or **1d**) (2.45 mmol/kg, ip) was administered at 0.5, 2, 4, or 8 h after an LD_{90} dose (4.97 mmol/kg, ip) of acetaminophen, and the survival rates of the animals were observed over 48 h. *The 25 animals in this group were normalized to 12 for comparison in this figure. †At the time of administration of **1b** at 8 h, there were only eight surviving animals. Thus, 2 out of 8 (25%) survived after drug treatment. ‡At the time of administration of **1d** at 8 h, there were only seven surviving animals. Thus, 4 out of 7 (57%) survived after drug treatment.

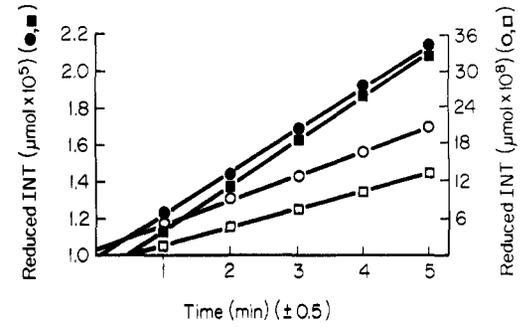
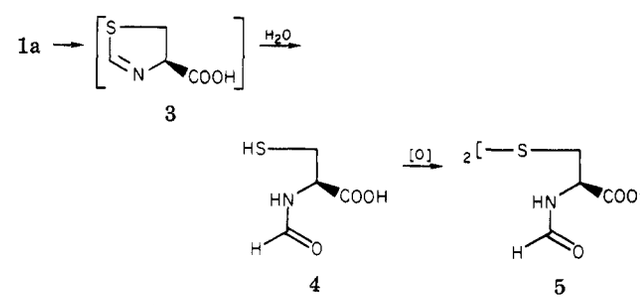


Figure 2. The rate of formation of reduced INT from **1b** with (○) and without (□) rat liver proline oxidase and from **7** with (●) and without (■) enzyme. See Experimental Section for details of the enzyme assay.

Scheme I



Compounds **1b** and **1d** were slightly less effective when administered 4 h after acetominophen, but they were fully protective when given 2 h after acetaminophen as when given by the usual protocol, i.e., 0.5 h after acetaminophen (Figure 1A,B). No protection was evident when **1b** was administered 8 h after the LD_{90} dose of acetominophen (Figure 1A). However, 4 of the 12 animals had already died by this time, and it is likely that liver damage had already reached maximum. Nevertheless, with the 2-*n*-propyl analogue, **1d**, a 57% survival rate was noted for the seven animals that received this compound at 8 h (Figure 1B). Although the survival rates with **1b–f** superimposed on lower doses of acetaminophen were not determined, the present results amply demonstrate that these 2-substituted thiazolidine-4(*R*)-carboxylic acids are highly effective in preventing toxic deaths elicited by large doses of acetaminophen. Moreover, the 2-methyl (**1b**), 2-*n*-propyl (**1d**), and 2-*n*-pentyl (**1e**) derivatives were nearly equipotent in preventing liver necrosis (Table I).

Metabolism of Thiazolidine-4-carboxylic Acids by Rat Liver Proline Oxidase. It is known[2] that thiazolidine-4(*R*)-carboxylic acid (**1a**) is metabolized by rat liver mitochondria to *N*-formylcysteine (**4**) and to *N,N'*-diformylcystine (**5**), the latter arising by oxidation of the former (Scheme I). The intermediate in this reaction was shown to be Δ^2-thiazoline-4(*R*)-carboxylic acid (**3**), which, on hydrolysis, yielded the products isolated. We now show that this oxidative biotransformation is catalyzed by proline oxidase, since **1a** was a substrate for the partially purified preparation of this enzyme from rat liver mitochondria with a measured K_m of 1.1×10^{-4} M and a V_{max} of 5.4 μmol min^{-1} (mg of protein)$^{-1}$. The K_m for L-proline, the natural substrate for this enzyme was determined to

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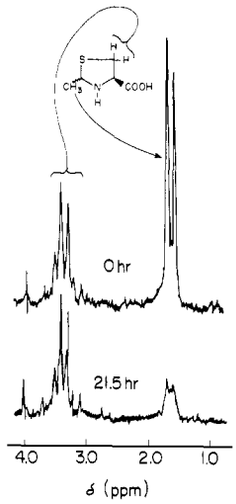
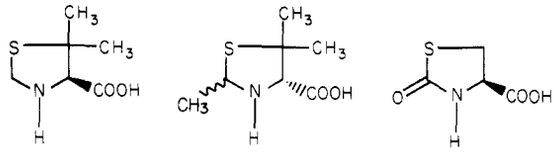


Figure 3. Proton NMR spectrum of **1b** in 0.1 M deuterated potassium phosphate buffer, pD 7.41, temperature 34 °C, at zero time and at 21.5 h showing the diminution in the intensities of the methyl doublet due to exchange with deuterium.

be 2.6×10^{-4} M, somewhat lower than reported.[3] The 4*S* enantiomer (**2a**) was not a substrate for proline oxidase, nor was 5,5-dimethylthiazolidine-4(*R*)-carboxylic acid (**6**) or its 4*S* enantiomer. It was surprising therefore that



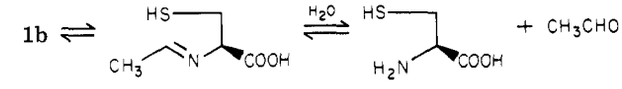
6 **7** **8**

incubation of either **1b** or 2(*RS*),5,5-trimethylthiazolidine-4(*S*)-carboxylic acid (**7**), a urinary pseudometabolite of ethanol isolated from D-penicillamine-treated rats,[4] with proline oxidase resulted in apparent oxidation of these substances (Figure 2). However, the colorimetric assay gave a positive response even in the *absence* of enzyme (Figure 2), leading to the suspicion that a nonenzymatic reaction was taking place.

Nonenzymatic Ring Opening of 2-Substituted Thiazolidine-4-carboxylic Acids. Riemschneider and Hoyer[5] have noted that 2-substituted thiazolidine-4-carboxylic acids undergo solvolysis with ring opening in aqueous solutions to uncover free sulfhydryl groups which can be titrated. The dissociation constants depended on the nature of the substituent at the 2-position of the thiazolidine ring and the pH of the solution. It is also known that the hydrogens on the methyl group at C-2 of **1b** undergo deuterium exchange in strongly alkaline solutions with $t_{1/2}$ of 3.2 h in a first-order reaction.[6]

We have observed that this deuterium exchange can also take place at physiological pH and temperature. When a solution of **1b** in D_2O or in 0.1 M deuterated potassium phosphate buffer, pD 7.41,[7] temperature 34 °C, was examined by NMR, the methyl proton doublet (actually, a pair of doublets when examined at higher resolution due to the presence of C-2 epimers) centered at δ 1.67 gradually disappeared over time due to deuterium exchange. The rate of this deuterium exchange could be measured quantitatively by comparing the diminution in integrated intensities of these methyl protons to the intensities of the C-5 methylene protons centered at δ 3.43 (AB multiplet of an ABX system), which integrated as a constant over time (Figure 3). Only deuterium exchange of the methyl protons was observed under these conditions, and the solvolysis products (Scheme II) were not detected. Cysteine exhibits a characteristic doublet (J = 6 Hz) at δ 3.07 and a triplet at δ 3.98, which were clearly missing (Figure 3). The first-order rate constant for this deuterium exchange was calculated to be 2.5×10^{-5} s^{-1} with a $t_{1/2}$ of 7.7 h. Addition of L-cysteine to this system at one-fourth the weight concentration of **1b**, however, slowed down this exchange considerably to the $t_{1/2}$ of 160 h. Since this deuterium exchange can only take place via a ring-opened Schiff base intermediate such as **9** (Scheme II), it is clear that the apparent "oxidation" of **1b** and **7** observed with proline oxidase (above) was an artifact due to nonenzymatic opening of the thiazolidine ring system. This was corroborated by the observation that added L-cysteine under the conditions of the colorimetric assay for proline oxidase (see Experimental Section) gave identical colorimetric responses. Although deuterium exchange was not observed with **7**, it is known that epimerization at the C-2 position of **7** takes place in neutral protic solvents, suggesting that **7** likewise undergoes ring opening under these conditions.[8] Deuterium exchange was also observed with the 4*S* enantiomer, **2b** ($k_1 = 2.9 \times 10^{-5}$ s^{-1}).

Scheme II



9

Table II. Excretion of $^{14}CO_2$ in Expired Air of Mice after Administration of [^{14}C]**1b**

time period, h	% of dose excreted as $^{14}CO_2$ [a]
0-4	61.9
4-8	7.0
8-12	4.3
12-16	2.9
16-24	3.7
	total: 79.8

[a] Averaged from two separate experiments.

Metabolism of 2-[^{14}C]Methyl[2-^{14}C]thiazolidine-4-(*R*)-carboxylic Acid to $^{14}CO_2$. Since **1b** is not a substrate for proline oxidase but instead dissociates at physiological pH to acetaldehyde and L-cysteine (Scheme II), administration of **1b** to mice should give rise to metabolic products that reflect this dissociation. Accordingly, the acetaldehyde formed in vivo should be oxidized to acetate by the aldehyde dehydrogenases of liver, and the acetate, in turn, should be metabolized along normal pathways, ultimately to CO_2. It follows that an equimolar quantity of L-cysteine must also be liberated during this process.

2-[^{14}C]Methyl[2-^{14}C]thiazolidine-4(*R*)-carboxylic acid ([^{14}C]**1b**) was prepared by condensation of [1,2-^{14}C]acetaldehyde and L-cysteine. A sample of [^{14}C]**1b** diluted with

(3) (a) 4.7×10^{-3} M. Dashman, T.; Lewinson, T. M.; Felix, A. M.; Schwart, M. A. *Res. Commun. Chem. Pathol. Pharmacol.* **1979**, *24*, 143. (b) 2.9×10^{-3} M. Kramar, R.; Fitscha, P. *Enzymologia* **1970**, *39*, 101.
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carrier was administered to mice, and the expired $^{14}CO_2$ was collected and monitored for radioactivity over 24 h. The radioactive label on [^{14}C]**1b** rapidly appeared as $^{14}CO_2$ in the expired air, nearly 62% of the administered dose being accounted for in this form after 4 h (Table II). The rate of $^{14}CO_2$ excretion rapidly diminished thereafter; nevertheless, after 24 h, a cumulative 80% of the administered radioactive dose was accountable as $^{14}CO_2$. These results indicate that [^{14}C]**1b** did, in fact, dissociate to [1,2-^{14}C]acetaldehyde in vivo and that the latter was rapidly metabolized to $^{14}CO_2$.

Discussion

Thiazolidine-4(*R*)-carboxylic acid (**1a**) has been used clinically in Europe for the treatment of hepatic and biliary disorders.[9] Compound **1a** has also been reported to protect mice against experimentally induced bromobenzene hepatotoxicity[10] and rats against acetaminophen-induced mortality.[10b,11] Although the mechanism of this protection by **1a** is not known, it is believed to be due to the uncovering of its masked sulfhydryl group in vivo.[12] This, of course, requires prior metabolism of **1a**.

More recently, **1a** has been reported to cause reverse transformation of tumor cells and to be effective for the treatment of epithelial tumors of the head and neck.[13] This has subsequently been challenged by a number of independent investigators;[14] moreover, the CNS toxicity of **1a** has been recognized and widely disseminated.[12,14a,15] It is believed, paradoxically, that this toxicity manifested by **1a** is due to *N*-formylcysteine or one of its oxidation products, *N*-formylcysteinesulfinic acid or *N*-formylcysteic acid,[12] although this has not been proven. It is recalled (Scheme I) that *N*-formylcysteine is produced metabolically by solvolysis of the intermdiate Δ^2-thiazoline-4(*R*)-carboxylic acid (**3**) formed in the oxidation of **1a** by liver mitochondrial proline oxidase.

N-Acetyl-L-cysteine (**10**) is also known to protect mice against acetaminophen-induced hepatotoxicity[16] and is presently the drug of choice for the clinical management of acetaminophen overdoses in humans. We had previously postulated[17] that the effectiveness of **10** was due not to **10** per se but to its hydrolysis product, L-cysteine, and possible conversion of the latter to glutathione. In support of this, **10** is known to be rapidly hydrolyzed to L-cysteine in the liver when administered in rats.[18] Moreover, (a) liver glutathione levels of mice depleted of this coenzyme by prior treatment with acetaminophen rebounded to twice that of a control group 6 h after administration of **10**,[19] and (b) ^{35}S-labeled **10** was shown to be incorporated into the glutathione–acetaminophen conjugate *prior* to conversion to the mercapturic acid conjugate.[20] It is noteworthy that 2-oxothiazolidine-4(*R*)-carboxylic acid (**8**), which is a good substrate for and a competitive inhibitor of 5-oxoprolinase, (a) is also metabolized to L-cysteine in vivo, (b) stimulates hepatic glutathione synthesis, and (c) protects mice against mortality elicited by high doses of acetaminophen.[21]

In contrast to the above, **1b** does not require metabolism to furnish L-cysteine in vivo; moreover, it is much less toxic than **1a**.[1] At doses comparable to **1a**, **1b** (as well as **1d** and **1e**) protected mice equally as well as **10** against histologically verified hepatic necrosis induced by acetaminophen (Table I).

The likely protective mechanism is as follows: administration of **1b–e** to mice delivers these sulfhydryl-masked prodrug forms of L-cysteine in vivo, releases this amino acid intracellularly by nonenzymatic ring opening followed by solvolysis per Scheme II, and stimulates the synthesis of hepatic glutathione. Delivery of L-cysteine to the liver and its subsequent incorporation into glutathione and *not* the liberation of sulfhydryl groups per se appear to be important in this protective mechanism, since **2b**, which dissociates to D-cysteine, was totally ineffective (Table I), as was DL-*N*-acetylpenicillamine, an analogue of **10** that is not deacetylated.[17] Interestingly, although the dissociation constants reported for **1b–d** at pH 6.3 and 40 °C are of the same order of magnitude (2.36, 4.96, and 1.83 $\times 10^{-5}$ M, respectively),[5] the 2-ethyl analogue **1c**—which gives rise to an odd carbon aldehyde—was much less protective than **1b** or **1d** (Table I). This suggests, possibly, that other in vivo factors may be affecting this dissociation.

In summary, we have shown that 2-substituted thiazolidine-4(*R*)-carboxylic acids—especially those derived from the condensation of L-cysteine with even carbon alkyl aldehydes, i.e., aldehydes that are readily metabolizeable to endogenous or other nontoxic products—can serve as prodrugs of L-cysteine and can protect mice against acetaminophen toxicity. Thus, a wide variety of prodrugs are available that are potentially capable of releasing L-cysteine in vivo, either by nonenzymatic solvolysis or by metabolic action. Since L-cysteine delivered to the liver stimulates hepatic glutathione biosynthesis and since glutathione is a required cofactor for the glutathione *S*-transferases of liver that conjugate and detoxify reactive metabolites generated in the oxidative biotransformation of xenobiotic substances,[22] these *cysteine prodrugs* should be effective antidotes for those xenobiotic substances that are metabolized to highly reactive and toxic intermediates.

Experimental Section

Melting points were determined on a Fisher-Johns melting point apparatus and are corrected to reference standards. Optical rotations were measured in a Perkin-Elmer Model 141 polarimeter, and radioactivity was counted in a Packard Tri-Carb Model 3255 liquid scintillation spectrometer. [1,2-^{14}C]Acetaldehyde was

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purchased from New England Nuclear Corp. Boston, MA. The following analytical instruments were used: IR, Beckman IR-10; UV–visible, Beckman Acta MVI; NMR, Varian T-60A.

2(*RS*)-Methylthiazolidine-4(*R*)-carboxylic Acid (1b). L-Cysteine (free base, 5.60 g, 0.046 mol) was suspended in 25 mL of H_2O in a 125-mL Erlenmeyer flask. To the cold (ice bath), stirred mixture was added, at once, 3.0 mL (2.20 g, 0.050 mol) of cold acetaldehyde, and the reaction flask was stoppered to prevent escape of the volatile reagent. The cysteine crystals dissolved within a few minutes, although the reaction mixture remained slightly opalescent. After 1 h of stirring in the cold, the reaction mixture was clarified by filtration through a medium pore sintered glass funnel, and the filtrate was diluted to 250 mL with absolute EtOH to initiate precipitation of the product. (The insoluble material removed by filtration was L-cystine which was confirmed by comparison of its IR spectrum with authentic material.) After standing several hours in a refrigerator, the mass of solids was broken up with a spatula, and the solids were allowed to age overnight in the cold. The product was collected by suction filtration on a coarse pore sintered glass funnel, squeezing out the excess solvent. When very little additional solvent could be removed by this procedure, the filter cake was repeatedly washed by suspending the solids in ether, followed by suction filtration. The filter cake was broken up with a spatula and dried in air initially and then in a vacuum desiccator with continuous vacuum pump action overnight to give 5.60 g (83% yield) of colorless microneedles, mp subl >140 °C, 166–168 °C dec (–gas) (reported[23] mp 161–163 °C dec); NMR (D_2O downfield from DSS) δ 1.65 and 1.68 (2 d, J = 7 Hz, 3 H, CH_3), 3.43 (ABX multiplet, 2 H, CH_2), 4.31 (t, J = 6 Hz, 1 H, H-4), 4.95 (br q, J = 7 Hz, 1 H, H-2). The sample also showed a trace of a methyl triplet at δ 1.07 and a multiplet at δ 3.70 due to EtOH; $[\alpha]^{23}_D$ –143.7° (*c* 1.01, H_2O). Anal. ($C_5H_9NO_2S$) C, H, N.

2(*RS*)-Methylthiazolidine-4(*S*)-carboxylic acid (2b) was prepared on a 0.02-mol scale with D-cysteine in the above procedure to give **2b** in 62% yield: mp subl >140 °C, 165–167 °C dec (–gas); $[\alpha]^{24}_D$ + 143.0° (*c* 1.0, H_2O). Anal. ($C_5H_9NO_2S$) C, H, N.

Rates of Deuterium Exchange of the Methyl Hydrogens of 1b and 2b in Deuterated Phosphate Buffer. The thiazolidinecarboxylic acid (0.05 g) was dissolved in 0.6 mL of potassium deuteriophosphate buffer, pD 7.41 (prepared by mixing 0.1 M KD_2PO_4 and 0.1 M K_2DPO_4 in D_2O). Deuterium exchange was followed at 34 °C by comparing the area of the methyl doublet centered at δ 1.67 ppm (downfield from DSS) with the multiplet centered at δ 3.43 ppm (Figure 3). The first-order rate constant for this exchange was determined from the slope of the line obtained by plotting log ($A_t - A_\infty$) vs. time, where A_t = area ratio at time t, and A_∞ = area ratio at 7 days.

2(*RS*)-[^{14}C]Methyl[2-^{14}C]thiazolidine-4(*R*)-carboxylic Acid ([^{14}C]1b). To a cooled, stirred solution of L-cysteine (122 mg, 1.00 mmol) in 10 mL of H_2O was added at once [1,2-^{14}C]-acetaldehyde (5.5 mg, 0.13 mmol, 8.0 mCi/mmol) in 1.0 mL of H_2O, followed by 6.3 mL (39 mg) of a cold aqueous stock solution of unlabeled carrier acetaldehyde containing 306 mg in 50 mL of H_2O. After the solution was stirred for 1 h in the cold and 2 h at room temperature, the solvent was evaporated in vacuo, and the residue was dried in a vacuum desiccator. Recrystallization of the product from methanol gave 100 mg (68.0% yield) of [^{14}C]1b, mp 163–164 °C dec. The specific radioactivity of this sample was determined to be 0.67 mCi/mmol, lower than the calculated specific radioactivity of 1.0 mCi/mmol based on dilution, very likely due to polymerization of part of the [1,2-^{14}C]acetaldehyde to its trimer, paraldehyde.

Metabolism of [^{14}C]1b to $^{14}CO_2$ by the Mouse. A mixture of [^{14}C]1b (0.236 mg, 1.04 × 10^{-3} mCi) prepared above and unlabeled **1b** (9.97 mg, 0.0677 mmol) dissolved in 0.7 mL of sterile H_2O was administered ip to a male, Swiss-Webster mouse (27 g, 2.59 mmol/kg). The animal was placed in an all-glass metabolism cage, and the expired $^{14}CO_2$ was collected in a trapping solution of ethanolamine/2-methoxyethanol (2:1, v/v). The trapping solution was replaced at 4, 8, 12, 16, and 24 h, and aliquots were counted for radioactivity by liquid scintillation counting.[24] An aqueous solution of $Na_2{}^{14}CO_3$ served as standard. This experiment was repeated with another mouse (24 g, 2.57 mmol/kg dose). The results are summarized in Table II.

Other 2-Substituted Thiazolidine-4(*R*)-carboxylic Acids (1c–g). 2(*RS*)-*n*-Propylthiazolidine-4(*R*)-carboxylic acid (1d) [mp 167–168 °C with dec; $[\alpha]^{30}_D$ –157° (*c* 0.39, 2% Na_2CO_3) reported mp 167–168[25] and 168–169 °C dec;[23] $[\alpha]^{20}_D$ –160° (c 0.5, 0.05 M borax, pH 8.80[5])] and **2(*RS*)-phenylthiazolidine-4-(*R*)-carboxylic acid (1f)** [mp 157–159 °C dec; $[\alpha]^{30}_D$ –132° (*c* 0.17, 2% Na_2CO_3) (reported mp 159–160[25] and 159–160 °C dec[23])] were prepared according to Schubert.[25] **2(*RS*)-Ethylthiazolidine-4(*R*)-carboxylic acid (1c)** [mp 162–163 °C dec: $[\alpha]^{31}_D$ –172° (*c* 0.19, 2% Na_2CO_3) (reported[23] mp 160–161 dec; $[\alpha]^{20}_D$ –175° (c 0.5, 0.05 M borax, pH 8.77[5])], **2(*RS*)-*n*-pentylthiazolidine-4(*R*)-carboxylic acid (1e)** [mp 157–158 °C dec; $[\alpha]^{32}_D$ –136° (*c* 0.13, 2% Na_2CO_3) (reported[23] mp 156–158 °C dec), and **2(*RS*)-(4-pyridyl)thiazolidine-4(*R*)-carboxylic acid (1g)** [mp 156–158 °C dec; $[\alpha]^{32}_D$ –173° (*c* 0.19, 2% Na_2CO_3) (reported[23] mp 160–162 °C dec; $[\alpha]^{20}_D$ –178° (*c* 0.5, 0.05 M borax, pH 8.87[5]) were prepared by adapting the procedure for the preparation of **1b.**

5,5-Dimethylthiazolidine-4(*R*)-carboxylic Acid (6). (Adapted from the procedure for the preparation of **7**.[4b]) L(+)-Penicillamine (5.0 g, 0.034 mol) was dissolved in 50 mL of hot H_2O, and the turbid solution was clarified by filtration. The solution was then cooled to 0 °C (ice–salt bath), 5 mL of a 37% aqueous formaldehyde solution was added slowly, and the mixture was allowed to stir for 1 h in an ice bath and then at room temperature overnight. The solvent was then evaporated in vacuo, and the white solid obtained was recrystallized from methanol to give 4.70 g of **6** (87% yield) in two crops: $[\alpha]^{25}_D$ –137.7° (*c* 0.99, MeOH); IR (KBr) 3400 (NH), 2400 (broad, COOH dimer), 1730 (COOH), 1600 cm^{-1}; NMR (Me_2SO-d_6, downfield from Me_4Si) δ 1.16 (s, 3 H, CH_3), 1.52 (s, 3 H, CH_3), 3.26 (s, 1 H, CH), centered at 4.09 (dd, 2 H, CH_2, J_{AB} = 9 Hz), 6.43 (br s, 1 H, NH). The melting point (201–202 °C dec) of this product was at variance with that reported in the literature (mp 193–194 °C)[26] and corresponded to the reported melting point of the racemic compound (200–201 °C). However, the 4*S* enantiomer prepared from D-penicillamine and formaldehyde by the above procedure gave mp 202–204 °C dec, $[\alpha]^{25}_D$ +136.3° (*c* 0.99, MeOH).

Protection of Mice against Acetaminophen Toxicity. The efficacy of 2-substituted thiazolidine-4-carboxylic acids in protecting mice against hepatic necrosis and death after LD_{90} doses of acetaminophen (Table I) was assessed in Swiss-Webster mice that were fasted overnight (16 h) as described previously.[1] Except for **1b**, which was made up in sterile H_2O, the test compounds were all prepared as a 15 mg/mL solution in sterile 0.1 N aqueous $NaHCO_3$ (injection grade) and administered within 1 h of preparation. The pH of the resulting solutions ranged from 6.5 to 7.5. For experiments where the administration of the protective agents **1b** and **1d** were delayed (Figure 1), food was made available ad libitum immediately after the dose of acetaminophen. Water was available at all times. Further details are described in the footnotes to Table I and in the legend for Figure 1.

Isolation of a Solubilized Proline Oxidase from Rat Liver Mitochondria. The isolation procedure for liver mitochondria from six male Sprague–Dawley rats ranging in weight from 170 to 225 g was essentially the same as described by Greenwalt.[27] The final mitochondrial pellet was resuspended in the isolation medium such that each milliliter contained about 130 mg of protein as determined by the Biuret assay.[28] The suspension was stored at –20 °C in 1-mL aliquots for 4 days prior to use. An aliquot of the stored mitochondrial preparation corresponding to about 130 mg of protein was suspended in 17 mL of ice-cold

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extraction medium (consisting of Triton X-100, 16 mM, potassium phosphate, 50 mM, and EDTA, 1 mM)[29] and allowed to stand in the cold for 1 h. The suspension was then centrifuged at 105000*g* for 60 min, and the supernatant liquid was collected and used directly as the enzyme source.

Assay Methods. Enzyme Specific Activity. For the determination of the specific activity of the enzyme, the assay (see below) was conducted with 10 μmol of L-proline. One unit of activity is defined as that amount of enzyme that catalyzes the reduction of 1 μmol of INT [2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride] per minute. The specific activities of the enzyme preparations were usually 0.6–0.7 unit/mg, with one preparation giving 2.9 units/mg.

Determination of K_m and V_{max} Values. Proline oxidase activity was assayed by following the reduction of INT by added substrate at 500 nm and at 30 °C by using a molar extinction coefficient of 11.5×10^6 for reduced INT.[30] The assay medium consisted of 100 μmol of potassium phosphate bufer (pH 7.5), 1.5 μmol of KCN, 0.39 μmol of stabilized menadione, 0.5 mg of INT, varying amounts of substrate, 0.1 mL of the solubilized enzyme preparation, and water to give a final volume of 3.0 mL. The reaction was initiated by adding the enzyme preparation last. Blanks were made up identically but without substrates. The data were analyzed on a Hewlett-Packard desk-top computer programmed to graphically display the best straight line fit of Lineweaver–Burk plots by weighted regression analysis using Michaelis–Menten kinetics. The K_m and V_{max} values were then calculated.

With compounds **2a** and **6**, no observable changes in the absorbance at 500 nm were discernible even after 5 min of incubation. However, with compounds **1b** and **7**, this absorbance increased progressively with time even when an equal volume of H_2O was substituted for the enzyme (Figure 2). This nonenzymatic color production could be mimicked by substituting L-cysteine for the thiazolidinecarboxylic acid in the assay medium.

Acknowledgment. This work was supported by the Veterans Administration. We thank William E. Smith for excellent technical assistance.

Registry No. (2*R*,4*R*)-**1b**, 88855-01-0; (2*S*,4*R*)-**1b**, 88855-02-1; (2*R*,4*R*)-[^{14}C]**1b**, 88867-03-2; (2*S*,4*R*)-[^{14}C]**1b**, 88867-04-3; (2*R*,4*R*)-**1c**, 88855-05-4; (2*S*,4*R*)-**1c**, 88855-06-5; (2*R*,4*R*)-**1d**, 88904-06-7; (2*S*,4*R*)-**1d**, 88904-07-8; (2*R*,4*R*)-**1e**, 88855-07-6; (2*S*,4*R*)-**1e**, 88855-08-7; (2*R*,4*R*)-**1f**, 64970-78-1; (2*S*,4*R*)-**1f**, 59999-67-6; (2*R*,4*R*)-**1g**, 88855-09-8; (2*S*,4*R*)-**1g**, 88855-10-1; (2*R*,4*S*)-**2b**, 88855-03-2; (2*S*,4*S*)-**2b**, 88855-04-3; (*R*)-**6**, 72778-00-8; (*S*)-**6**, 22916-26-3; L-cysteine, 52-90-4; D-cysteine, 921-01-7; acetaldehyde, 75-07-0; [1,2-^{14}C]acetaldehyde, 1632-97-9; L-(+)-penicillamine, 1113-41-3; D-penicillamine, 52-67-5; formaldehyde, 50-00-0; butanal, 123-72-8; benzaldehyde, 100-52-7; propanal, 123-38-6; hexanal, 66-25-1; 4-pyridinecarboxaldehyde, 872-85-5; acetaminophen, 103-90-2.

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Synthesis of Pyridine Derivatives of L-Phenylalanine as Antisickling Reagents

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Several bicyclic agents composed of L-phenylalanine coupled to various pyridines were synthesized: 2-, 3-, and 4-(L-phenylalanylamino)pyridine. All three compounds at 3 mM gave positive morphological antisickling effects on homozygous SS cells under reduced O_2 tension. Studies on two of these compounds, 2- and 3-(L-phenylalanylamino)pyridine, showed that these agents increase the deoxy-HbS solubility ratio, C_s/C_s^0, by 14% at 20 mM. Observed changes in the mean corpuscular hemoglobin concentration (MCHC) values of treated cells ranged from 4% at 1.3 mM to 15% at 5.6 mM in compound concentration. Very minor lytic activity was found for treated cells, indicating water uptake is responsible for changes in the MCHC. Further, exposure of sickle cells to a 3 mM concentration of these agents also increased by 6- to 7-fold cellular deformability of a treated erythrocyte population as compared to an untreated one at the same total O_2 saturation of 47%. These agents demonstrate the potential of bicyclic compounds composed of a common constituent, L-Phe, in the development toward a viable therapeutic agent.

Deoxygenated sickle cell hemoglobin, deoxy-HbS, has been shown to have increased solubility in the presence of aromatic amino acids,[1,2] oligopeptides,[3-5] alkylureas,[6] different phenyl derivatives,[7-12] aryl-substituted alanines,[13] and small aromatic di- and tripeptides.[14] Little is known concerning the mechanism by which noncovalent interactions betwen these compounds and the HbS tetramer result in increased HbS solubility. However, hydrophobicity, a measure of the nonpolar content of these noncovalent antigelling agents, is an important chemical feature. It is related to their antigelling activity as measured by the concentration of the compound required in solution to delay gelation or increase the minimum gelling concentration of deoxy-HbS. With respect to aromatic compounds, bicyclic agents have been shown[13,14] to be more effective than monocyclic members. Increasing the polarizability of the ring system via halogenation also plays a role in enhancing the antigelation activity of aromatic compounds.[10,13] Two different approaches have been re-

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