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MICROSOMAL OXIDATION OF α-THIOCARBOXYLIC ACIDS TO SULFOXIDES*

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Abstract—Five carboxyl-labeled ^{14}C-α-thiocarboxylic acids were synthesized by reaction of carboxyl-^{14}C-iodoacetate with ethyl, propyl, isopropyl, phenyl and benzyl mercaptans. These compounds were found to be oxidized to the corresponding sulfoxides by the microsomal fraction from rat liver homogenates. The system required an NADPH-regenerating system and O_2 and is optimal at pH 7·4. The oxidative reaction proceeds in a linear fashion at 37° until substrate is nearly completely converted; K_m for S-carboxymethylethyl mercaptan is 8×10^{-4} M.

STUDIES on the biological oxidation–reductions of sulfides, sulfoxides and sulfones are prerequisite to an understanding of the metabolism of many important natural and synthetic compounds. Among these are some of the L-amino acids, a few vitamins, certain drugs, solvents, and even some toxic compounds.

Work has been done on the biological oxidation of sulfur-containing amino acids and a few thioethers *in vivo* and *in vitro*. For example, the formation of sulfate from dimethylthetin and ethylthetin has been reported,[1,2] as has sulfoxide formation in the metabolism of S-*n*-propyl-L-cysteine by rat liver microsomes,[3] S-methyl-L-cysteine by intact leaves,[4,5] phenothiazine by calves and sheep,[6] and 4-(phenyl-thioethyl)-1,2-diphenyl-3,5-pyrazolidinedione in man.[7] Moreover, thiophenol is oxidized to sulfone as a key detoxication mechanism in rats.[8] Also, Maw[1] and Young and Maw[9] have shown that methylthioacetate, ethylthioacetate and mercaptoacetate were oxidized by liver slices.

The purpose of the present investigation was to study the possibility of the general enzyme-catalyzed oxidation of α-thiocarboxylic acids by preparations from rat liver. The results shown in this paper indicate that several chemically synthesized α-thiocarboxylic acids are oxidized to different extents to their sulfoxide derivatives by an electron transport system requiring NADPH and O_2 and located in microsomes.

EXPERIMENTAL

Commercial materials. NADPH and NADH were purchased from Sigma Chemical Company. DL-Isocitrate (trisodium salt) and isocitric dehydrogenase were from Calbiochem. Mercaptans were from Eastman Organic Chemicals. Carboxyl-labeled ^{14}C-iodoacetic acid was from Tracerlab.

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Synthesis of ^{14}C-α-thiocarboxylic acids. Five carboxyl-labeled ^{14}C-α-thiocarboxylic acids, i.e. S-carboxymethylethyl mercaptan, S-carboxymethylpropyl mercaptan, S-carboxymethyl-*i*-propyl mercaptan, S-carboxymethylphenyl mercaptan, and S-carboxymethylbenzyl mercaptan, were prepared by a modified Williamson synthesis[10] by reaction of sodium iodoacetate-^{14}C with the appropriate mercaptan in dilute NaOH. For this, the mercaptan (0·25 mole equivalents) was dissolved in 250 ml of 10 per cent NaOH and added to iodoacetic acid (0·25 mole equivalents) dissolved in 250 ml water. After 2 hr at room temperature, the solution was adjusted to pH 8 with 5 N HCl and extracted with an equal volume of diethyl ether to remove any unreacted mercaptan. The aqueous phase was acidified to below pH 1 and extracted twice with 250 ml ether to obtain crude product. The combined ether phases were rinsed by back-extraction with an equal volume of water, dried over anhydrous Na_2SO_4, and evaporated to dryness for the S-carboxymethylalkyl mercaptan.

After weighing to obtain yields, an aliquot of each of the liquids was again weighed to calculate specific gravity. Melting points of the solids were obtained with a Fisher–Johns apparatus.

Cell fractions. Adult male, Sprague–Dawley rats, weighing 250–300 g, were killed by rapid decapitation and their livers immediately removed and homogenized in 4 vol. of cold 0·1 M potassium phosphate buffer, pH 7·4. The subsequent operations were performed at 0°–4°. The homogenate was centrifuged successively at 700 *g* for 5 min to sediment the cell debris and nuclei, at 10,800 *g* for 20 min to sediment the mitochondria, and at 105,000 *g* for 1 hr to sediment the microsomes and leave the supernatant solution. All the cellular fractions were resuspended in 0·1 M potassium phosphate buffer to make each of their final volumes equal to the original volume of homogenate.

Incubation mixtures and assays. Unless otherwise indicated, a typical incubation mixture in a 50-ml Erlenmeyer flask consisted of 2·5 μmoles carboxyl-labeled ^{14}C-α-thiocarboxylic acid (most commonly the S-carboxymethylethyl mercaptan), 1·2 μmoles NADPH, 120 μmoles DL-isocitrate, 1·25 units isocitric dehydrogenase, 2·5 ml of cell fraction (equivalent to 0·5 g tissue), and 2·5 ml of 0·1 M potassium phosphate buffer, pH 7·4, to a final volume of 5 ml. The mixture was incubated with shaking for 1 hr at 37°, the reaction was stopped by the addition of 1·5 ml of 1 N HCl, and then centrifuged to remove the precipitate. To the supernatant, an equal volume (5 ml) of 95 per cent ethanol was added. The turbid solution was again centrifuged, and the supernatant obtained was extracted three times with 10-ml portions of ether. The combined ether extract was evaporated to dryness and the residue dissolved in 1·5 ml ether. Radioactivity was determined for a 0·1-ml aliquot in 10 ml of Bray's solution[11] with a Packard Tri-Carb liquid scintillation spectrometer. Over 90 per cent of the total radioactivity was recovered. An exact portion (50 μl) was applied to Whatman No. 1 paper for ascending chromatography with *n*-butyl alcohol–acetic acid–water (2:1:1, v/v) as solvent. The sheets were developed for 24 hr, cut into 1·5-in. strips, and scanned for radioactivity with a Nuclear-Chicago model 1002 radiochromatogram strip scanner. The R_f values were calculated from the positions of the compounds and the percentage conversion of the substrate obtained by calculating the areas of the peaks on the radiochromatogram.

To measure possible decarboxylation of the ^{14}C-substrates, incubations were also done in 50-ml flasks stoppered with a rubber septum to which was appended a poly-

propylene center well containing a filter-paper wick soaked in 0·5 ml of alkaline Hyamine solution. Reactions with whole liver homogenates were terminated with acid after 1 hr of incubation as before. The flasks were allowed to shake for an additional 15 min to ensure that all the $^{14}CO_2$ was adsorbed onto the filter paper. The center well and wick was snipped from the septum and dropped into a toluene counting solution for determination of radioactivity in the liquid scintillation counter. Also, the incubation mixture was centrifuged, the pellet rinsed with ethanol, and both solutions were filtered through a Millipore filter. After reducing the volume of the combined filtrates and recording the volume, a 0·5-ml aliquot was counted in 10 ml of Bray's solution.

Isolation of the metabolite from S-carboxymethylethyl mercaptan. An incubation mixture comprised of the same proportions of ingredients as those used for assay, but in 20-fold larger amounts, was made up to contain the ^{14}C-S-carboxymethylethyl mercaptan and microsomes. After incubation for 1 hr, acidification, and centrifugations as before, the supernatant obtained was extracted three times with 100-ml portions of ether. The combined ether extract was evaporated to dryness and the residue dissolved in 10 ml ether. This solution was streaked on a sheet of Whatman No. 3 paper to which a reference standard of the ^{14}C-substrate had been applied, and the chromatogram was developed with the butanol–acetic acid–water solvent. The radioactivity not corresponding to the standard was eluted from the excised strip with 10 ml of glacial acetic acid which was then removed by evaporation under reduced pressure.

RESULTS

Characteristics of ^{14}C-α-thiocarboxylic acids. The yields obtained and specific gravities or melting points of the synthetic α-thiocarboxylic acids are listed in Table 1.

TABLE 1. YIELDS, SPECIFIC GRAVITIES AND MELTING POINTS OF α-THIOCARBOXYLIC ACIDS

Compound	Yield (%)	Specific gravity	Melting point (°C)
$CH_3CH_2SCH_2CO_2H$	93	1·137	
$CH_3(CH_2)_2SCH_2CO_2H$	75	1·093	
$(CH_3)_2CHSCH_2CO_2H$	91	1·097	
$C_6H_5SCH_2CO_2H$	97		57–59*
$C_6H_5CH_2SCH_2CO_2H$	96		58–62

* Reported as 61–62.[12]

All compounds were judged to be pure, as single spots were observed in each case when the α-thiocarboxylic acids were chromatographed on silica gel sheets (Brinkmann N-HR) with the ascending butanol–acetic acid–water solvent and compounds detected by brief exposure of the chromatogram to iodine vapor.

Elemental analyses of the α-thiocarboxylic acids, done by Schwarzkopf Microanalytical Laboratory of Woodside, N.Y., are reported in Table 2.

Nature of the conversion of α-thiocarboxylic acids by liver preparations. The possibility that decarboxylation or other fragmentation of the carboxymethyl portions

TABLE 2. MICROANALYSES OF α-THIOCARBOXYLIC ACIDS

Compound	C	H	O	S
$CH_3CH_2SCH_2CO_2H$				
Calculated	39·97	6·72	26·63	26·68
Found	40·07	6·98	26·77	26·57
$CH_3(CH_2)_2SCH_2CO_2H$				
Calculated	44·74	7·59	23·84	23·83
Found	44·76	7·80	23·85	23·62
$(CH_3)_2CHSCH_2CO_2H$				
Calculated	44·74	7·59	23·84	23·83
Found	44·26	7·98	23·81	23·66
$C_6H_5SCH_2CO_2H$				
Calculated	57·11	4·80	19·02	19·06
Found	57·07	5·01	19·07	19·37
$C_6H_5CH_2SCH_2CO_2H$				
Calculated	59·31	5·54	17·56	17·60
Found	59·35	5·84	17·59	17·02

of the ^{14}C-α-thiocarboxylic acids may occur during incubation with liver homogenates was ruled out. No significant volatile, radioactive compound appeared in the Hyamine solution which would effectively trap CO_2 and much of any formate or acetate that could conceivably form. Radioactivity initially added was satisfactorily accounted for as remaining in the aqueous solution of the incubation mixture.

Incubation of the ^{14}C-S-carboxymethylethyl mercaptan ($R_f = 0{\cdot}71$) with the various cell fractions from rat liver led to formation of a ^{14}C-product with an R_f value of 0·81 upon paper chromatography in the butanol–acetic acid–water system. In contrast, no detectable ^{14}C-product was found using boiled cell fractions. The data presented in Table 3 indicate the intracellular distribution of activity responsible for such conversion of the α-thiocarboxylic acid. The microsomal fraction is seen to be most active and is even somewhat better than an equivalent amount of whole homogenate.

TABLE 3. CONVERSION OF S-CARBOXYMETHYLETHYL MERCAPTAN BY DIFFERENT CELL FRACTIONS*

Fraction	^{14}C-substrate converted (%)
Homogenate	24
Nuclear	1
Mitochondrial	7
Microsomal	38
Supernatant	6

* A 2·5-ml portion of the cell fraction equivalent to 0·5 g liver was incubated at 37° for 1 hr with 2·5 μmoles ^{14}C-carboxymethylethyl mercaptan ($1{\cdot}09 \times 10^5$ dis./min) in pH 7·4 phosphate buffer for a total of 5 ml, as described in Experimental.

The effects of pH and time on conversion of the S-carboxymethylethyl mercaptan by the microsomal fraction from rat liver are illustrated in Fig. 1. The optimal pH is between 7·0 and 7·5. Increase in product formation is a linear function of time up to approximately 1 hr, by which time the reaction is nearly complete.

The extent of product formation is typically related to substrate concentration for enzyme-catalyzed reactions. As calculated from the Lineweaver–Burk plot (Fig. 2), K_m for S-carboxymethylethyl mercaptan is 8×10^{-4} M.

Only a very small amount of product is observed in the presence of NADH and but little more with NADPH. However, the NADPH-regenerating system elicits marked stimulation of the reaction. The reaction is also dependent on molecular oxygen, since gassing the assay medium for 5 min with nitrogen before stoppering caused a 90–95 per cent inhibition of product formation.

A comparison of the specificity of the microsomal system to the five synthetic α-thiocarboxylic acids is indicated by the data in Table 4. S-Carboxymethylethyl

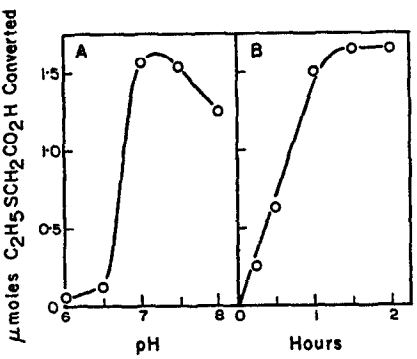

FIG. 1. The effects of pH (A) and incubation time (B) on the conversion of ^{14}C-S-carboxymethylethyl mercaptan by rat liver microsomes.

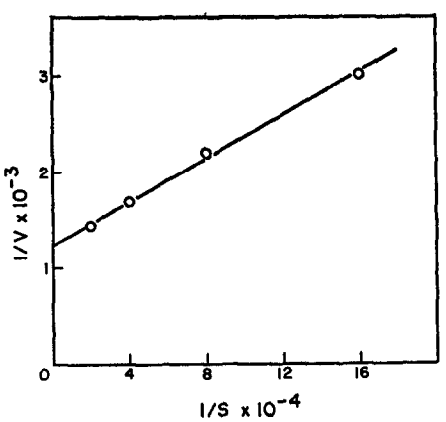

FIG. 2. The effect of substrate concentration on the conversion of ^{14}C-S-carboxymethylethyl mercaptan by rat liver microsomes.

TABLE 4. SPECIFICITY OF THE MICROSOMAL SYSTEM FOR α-THIOCARBOXYLIC ACIDS*

^{14}C-S-carboxymethylalkyl mercaptan	^{14}C-product (%)	Relative activity (%)†
Ethyl	36	100
Propyl	33	92
Isopropyl	31	87
Phenyl	27	75
Benzyl	8	23

* A 2·5-ml portion of the microsomal fraction equivalent to 0·5 g liver was incubated at 37° for 1 hr with 2·5 μmoles ^{14}C-S-carboxymethylalkyl mercaptan in pH 7·4 phosphate buffer for a total of 5 ml, as described in Experimental.

† The percentage of radioactive product formed from ^{14}C-S-carboxymethylethyl mercaptan ($1·09 \times 10^5$ dis./min) was considered as the 100 per cent reference to calculate the relative activity with other ^{14}C-S-carboxymethylalkyl mercaptans ($1·0$ to $1·1 \times 10^5$ dis./min).

mercaptan appears to have the greatest substrate activity, followed closely by the *n*-propyl and *i*-propyl derivatives. The aromatic substrates, especially the benzyl derivative, are less reactive.

Identification of the metabolite. A portion of the metabolite from the large-scale incubation of ^{14}C-S-carboxymethylethyl mercaptan was reduced by treatment at room temperature with zinc powder in glacial acetic acid. Also, a portion of the original substrate was oxidized in a standard manner[13] to the sulfoxide by equimolar H_2O_2 in glacial acetic acid. These compounds, viz. the original substrate, the metabolite and sulfoxide and reduced derivatives of these, were compared as to chromatographic mobilities indicated in Table 5. It is clear that the S-carboxymethylethyl mercaptan has an R_f value which is the same as that of the chemically reduced microsomal product, and the microsomal product has an R_f value coincident to that of the known sulfoxide of S-carboxymethylethyl mercaptan. Also, the ^{14}C-S-carboxymethylphenyl mercaptan was found to yield a product which, together with the carrier, synthetic

TABLE 5. CHROMATOGRAPHIC IDENTITY OF PRODUCT FROM MICROSOMAL OXIDATION OF S-CARBOXYMETHYLETHYL MERCAPTAN

^{14}C-compound	R_f (butanol–acetic acid–water)
S-Carboxymethylethyl mercaptan	0·71
Reduced microsomal product*	0·72
Microsomal product†	0·80
S-Carboxymethylethyl mercaptan sulfoxide	0·82

* Formed by treatment of microsomal product with zinc in acetic acid at room temperature.

† Formed by incubation of S-carboxymethylethyl mercaptan with liver microsomes for 1 hr at 37° and pH 7·4, as described in Experimental.

sulfoxide of this phenyl α-thiocarboxylic acid, approached constant specific radioactivity after repeated recrystallization. Thus, the product from the NADPH-dependent microsomal oxidation of an α-thiocarboxylic acid is the sulfoxide.

DISCUSSION

The experimental results presented herein demonstrate that an enzyme system located in rat liver microsomes and dependent upon NADPH and O_2 catalyzes the oxidation of α-thiocarboxylic acids to their sulfoxide analogs. These findings are in line with some other observations of the metabolism of thioethers in biological systems.[6,7] Gillette and Kamm[14] have shown that chlorpromazine and 4,4′-diaminodiphenyl sulfide are oxidized to their sulfoxides by an NADPH-dependent system located in guinea pig liver microsomes. NADPH and oxygen are also required by the enzyme system that catalyzes the oxidative demethylation of *N*-methylhydrazines in rat liver microsomes[15] and the hydroxylation of steroids and certain amino acids.[16] Hence, the common requirements for NADPH and oxygen are characteristic of the oxidative, electron transport system in microsomes related to sulfoxidation, demethylation and hydroxylation reactions.[17] The obligatory requirement for an NADPH-regenerating system to achieve maximal enzymic activity, rather than using NADPH alone, is due to the rapid oxidation of NADPH by microsomes even in the absence of added substrate. Moreover, the somewhat less activity seen with whole homogenate is likely due to the enzymes present in other fractions which catalyze oxidation and perhaps decomposition of the needed NADPH.

Brown and Scholefield[18] proposed that the β-oxidative metabolism of thio-fatty acids in rats results in the appearance of sulfate in urine. It may also be that the sulfoxide is but an intermediate in the further catabolism of α-thiocarboxylic acids. The lesser activities shown by phenyl and benzyl derivatives is in accordance with the view of Hill and Lewis[19] that aromatic bound sulfur is poorly oxidized, if at all. The reduction of sulfoxide to thioether also occurs in biological system. Black[20] has described an enzyme system prepared from yeast extracts which catalyzes reduction of L-methionine sulfoxide to L-methionine. Whether or not such a back-reaction can occur with sulfoxides of the presently investigated α-thiocarboxylic acids remains to be determined.

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