The Conjugation of Glutathione with Unsaturated Acyl Thiol Esters and the Metabolic Formation of *S*-Carboxyalkylcysteines

By T. W. SPEIR AND E. A. BARNESLEY

*Department of Biochemistry, Victoria University, Wellington, New Zealand,
 and Department of Biochemistry, University of Birmingham, Birmingham B15 2TT, U.K.*

(Received 11 June 1971)

The partial purification and properties of an enzyme from the soluble fraction of rat liver that catalyses the reaction of glutathione with 2,3-unsaturated acyl thiol esters is described, and its possible role in the formation of *S*-carboxyalkylcysteines is discussed. The synthesis of *S*-(3-methylcrotonyl)- and *S*-(2-methylcrotonyl)-*N*-acetylcysteamine and of *S*-crotonyl-*NN*-dimethylcysteamine hydrochloride and dicyclohexylammonium *S*-crotonyl-*N*-acetyl-L-cysteine is described.

S-Carboxyalkylcysteines occur in plant material and animal tissues, and are excreted in animal and human urine (for reviews see Fowden, 1964; Kodama, 1968). The structure of the *S*-substituent of many of these compounds suggests that they originate from the metabolites of certain amino acids. Fukutome (1961) observed the excretion of *S*-(1-carboxy-2-methylpropyl)cysteine by cats after the administration of leucine, and Suzuki, Sugii & Kakimoto (1962) observed the incorporation of [¹⁴C]valine into the *S*-substituents of *S*-(2-carboxypropyl)cysteine and *S*-(2-carboxypropyl)glutathione in garlic root. Isovaleric acid, which occurs as its CoA ester in the degradation pathway of leucine, also gave rise to *S*-(1-carboxy-2-methylpropyl)glutathione when incubated with guinea-pig liver homogenates containing added ATP and glutathione (Kuwaki, Ohmori & Mizuhara, 1963), but on the other hand Fukutome (1962*a,b*) observed that although the administration of isovalerate to several species was followed by an increased excretion of *S*-(1-carboxy-2-methylpropyl)cysteine, significant amounts of radioactivity were not incorporated into the amino acid when ¹⁴C-labelled isovalerate was used.

Boyland & Chasseaud (1967, 1968) have demonstrated that there are enzymes in rat liver that catalyse the addition of glutathione to the double bond of certain reactive alkenes not normally found in the animal body. This type of reaction between glutathione and naturally occurring 2,3-unsaturated acyl-CoA thiol esters or their partial hydrolysis products could account for the formation of intermediates, which on hydrolysis would yield *S*-carboxyalkylcysteines (Scheme 1 and Table 1). In this paper we present evidence for the activity of an enzyme that could catalyse these reactions.

MATERIALS

Preparation of S-acyl-N-acetylcysteamines. The preparation of this type of compound has been described in detail (Lynen, 1969). The procedure given below is much simpler, however, and not inferior with respect to final yield and purity. Cysteamine hydrochloride (11.4 g) in water (50 ml) was titrated at 0°C with 2*M*-NaOH until the pH of the solution had risen to 12. Acetic anhydride was added in 0.5 ml portions to the cooled stirred solution, and 2*M*-NaOH was added to maintain the pH at 12. The addition of 9.5 ml of acetic anhydride over a period of 1 h during which the temperature fluctuated between 3° and

Table 1. *Some S-carboxyalkylcysteines from animal sources and structurally similar metabolites from which the S-substituents might be derived*

<i>S</i> -Substituent of carboxy-alkylcysteine	2,3-Unsaturated acyl substituent of CoA derivative	Amino acid source of acyl-CoA
2-Carboxyethyl	Acryloyl	β-Alanine
2-Carboxypropyl	2-Methylacryloyl	Valine
2-Carboxy-1-methylethyl	Crotonyl	Lysine
2-Carboxy-1-methylpropyl	2-Methylcrotonyl (tiglyl)	Isoleucine
1-Carboxy-2-methylpropyl	3-Methylcrotonyl	Leucine
1,1-Di(carboxymethyl)methyl	Glutaconyl	Lysine

Scheme 1.

6°C was accompanied by the uptake of 52 ml of alkali to maintain the pH, and this indicated a fractional acetylation of 0.96. Then NaBH₄ (0.9 g) was added to the solution and after 1 h the solution was diluted to 1 litre with water and acetic acid was added dropwise to lower the pH to 7–8 and decompose the excess of NaBH₄. The solution was passed through a column (volume 300 ml) of Zeo-Karb 225 (H⁺ form) resin, and the column was washed with water until the pH of the effluent increased to 5. The pooled effluent was adjusted to pH 6.0 with 2M-NaOH, and a solution of lead acetate (19.0 g of the trihydrate) in water (150 ml) was added. The mixture was again treated with NaOH to adjust the pH to 6.0, and after it had been left overnight the yellow precipitate of lead *N*-acetylcysteamine mercaptide was collected by filtration. The precipitate was washed on the filter with water, and then stirred with two 400 ml portions of water and re-filtered. It was finally washed with acetone and dried over P₂O₅ *in vacuo*. The yield was 16.3 g (73%) [Found: N (by Kjeldahl), 6.3%; equiv. 224 (determined by dissolving weighed samples of about 50 mg in 10 ml of 5M-acetic acid, then adding 1 ml of 1M-KI, and titrating the solution to the starch indicator end point with 5mm-KIO₃). Calc. for C₈H₁₆N₂O₂PbS₂: N, 6.3%, equiv. 222].

N-Acetylcysteamine lead mercaptide (5 mmol) was stirred in dry ether (30 ml) with 10 mmol of acid chloride until the yellow colour of the mercaptide had disappeared. The solution was filtered and the residue was washed with ether. To the combined filtrate and washings cyclohexane or *n*-heptane was added until a slight turbidity appeared, and the solution was cooled to –25°C. The solution was decanted from the coloured oil that first separated and allowed to crystallize at –25°C. The yield of crude product was increased by recombining oil and mother liquors, evaporating off part of the ether, and repeating the crystallization. The crude products were combined and recrystallized from ether and cyclohexane, and the final yields varied between 20 and 35% (based on the mercaptide). The properties of the crotonyl, 2-methylcrotonyl, and 3-methylcrotonyl esters that had been prepared by this method are given in Table 2. Acryloyl and 2-methylacryloyl esters could not be prepared by this method; the products obtained appeared to be thiol esters of polyacrylic acids.

Preparation of dicyclohexylammonium S-crotonyl-N-acetyl-L-cysteine. *N*-Acetyl-L-cysteine (3.26 g) was dissolved in ethanol (100 ml), and the solution was brought to an apparent pH of 7.4 by the addition of 1M-dicyclohexylamine in ethanol. To the solution at 0°C crotonyl chloride (2.09 g) in dioxan (10 ml) was added, then 1M-dicyclohexylamine in ethanol was added to return the pH to 7.4. The reaction mixture was diluted to 1 litre with acetone, then kept at 0°C for 18 h, and filtered. The filtrate was evaporated to dryness under reduced pressure at 45°C, and the residue was fractionally crystallized from

solution in ethanol or chloroform by the addition of acetone. The distribution of the product between fractions was determined by measuring the *E*₂₆₀ of aqueous solutions, and repeated crystallization was required to obtain 1.02 g (12% yield) of the pure compound (Table 2).

Preparation of S-crotonyl-NN-dimethylcysteamine hydrochloride. *NN*-Dimethylcysteamine hydrochloride (1.42 g) in ethanol (30 ml) was stirred with about 30 ml of Amberlite IR 45 resin (acetate form) that had been washed with ethanol. To the cooled mixture (apparent pH 6.8) crotonyl chloride (1.05 g) in dioxan (10 ml) was added, and the mixture was stirred until the pH had risen to 5.8. Water (10 ml) was added, and after stirring for 15 min the mixture was filtered and the resin was washed with three 25 ml portions of ethanol. The filtrate and washings were evaporated at 50°C, and the residue obtained was dried over P₂O₅ *in vacuo*. The mixture of gum and crystals that resulted was extracted thoroughly with acetone (80 ml) containing conc. HCl (0.8 ml), and the suspension that was obtained was centrifuged. The clear supernatant was evaporated at 50°C, and the residue was crystallized, first from concentrated solution in acetone and then from solution in a mixture of acetone and chloroform by the addition of ether; yield 0.78 g (37%) (Table 2).

Preparation of S-crotonylpantetheine. Pantetheine (0.91 g) was dissolved in 10 ml of 1.5M-acetic acid and was then stirred with zinc dust. After 2 h the suspension was filtered, and the filtrate was passed through a column of Zeo-Karb 225 (H⁺ form) resin. The column was washed with water, and the total effluent was freeze-dried. The residue was dissolved in dioxan (4 ml), crotonyl chloride (0.38 g) in dioxan (6 ml) was added to the solution, and the acid that was liberated was titrated with 2M-NaOH. A pale-yellow oil separated. Most of the dioxan was removed by evaporation, and the oil was separated, dissolved in chloroform, and washed with water. The solution was chromatographed on Whatman 3MM paper with solvent A (see 'Chromatography'), the product was eluted from the paper with chloroform, and the extract was evaporated and dried over P₂O₅ *in vacuo*. The product, yield 0.52 g, had ε₆₅₀₀ in water at 263 nm.

Preparation of S-(2-carboxy-1-methylethyl)-, S-(1-carboxy-2-methylpropyl)- and S-(2-carboxy-1-methylpropyl)-L-cysteine. These compounds were prepared by published methods (Oomori & Mizuhara, 1962; Kodama, 1968). The amino acids were recovered from their reaction mixtures by adsorption on Zeo-Karb 225 (H⁺ form) resin, and displaced from the resin with dilute aq. NH₃. After evaporation to remove NH₃ the amino acids were adsorbed on De-acidite G or Amberlite CG-4B resin (acetate form, equilibrated with 0.1M-acetic acid), the resin was washed with 0.1M-acetic acid to displace neutral amino acids, and then with 2M-acetic acid to displace the acidic amino acids. The acidic amino acids were recovered from solution by evaporation and were

Table 2. Properties of synthetic 2,3-unsaturated acyl thiol esters

Compound	m.p.*	Wavelength of maximum extinction (nm)	$10^{-3} \times$ Extinction coefficient (l-mol ⁻¹ ·cm ⁻¹)	Elementary analysis (%)							
				Predicted				Found			
				C	H	N	S	C	H	N	S
S-Crotonyl-N-acetylcysteamine	62†	263	6.7†	51.3	7.0	7.5	17.1	51.1	7.1	7.7	17.0
S-2-Methylcrotonyl-N-acetylcysteamine	†	259	10.4	53.7	7.5	7.0	15.9	53.7	7.7	6.9	15.7
S-3-Methylcrotonyl-N-acetylcysteamine	50.5–51.5°C	266	11.7	53.7	7.5	7.0	15.9	53.8	7.6	7.0	15.7
S-Crotonyl-NN-dimethylcysteamine	146.5–147.5°C	256	6.47	45.8	7.7	6.7	15.3	45.8	7.9	6.6	15.1
hydrochloride											
Dicyclohexylammonium S-Crotonyl-N-acetyl-L-cysteine	163–164°C§	261	5.35	61.1	8.8	6.8	7.8	61.0	9.0	6.9	7.7
S-Crotonylpantetheine		263	6.5			4.2	9.7			4.0	9.6

* Not corrected.

† Lynen (1969) gives m.p. 62–63°C, ϵ 6500 at 263 nm.

‡ Melts below room temperature.

§ Melted with decomposition.

|| Product was a gum.

then crystallized from water or from aqueous acetone or ethanol. They were chromatographically and analytically pure.

Preparation of S-(2-carboxy-1-methylethyl)glutathione. Glutathione was reacted with crotonic acid by a method similar to that described by Ohmori, Shinomura, Azumi & Mizuhara (1965) for the preparation of the cysteine derivative. The product was purified by chromatography on Whatman 3MM paper with solvent A, and the major component (detected with ninhydrin at R_F 0.42) was eluted with water. The extract was evaporated at 45°C, and the residue was crystallized from acetic acid by the addition of acetone. The product represented only 32% of the glutathione used. (Found: C, 42.5; H, 6.0; N, 10.6; S, 8.0. Calc. for $C_{14}H_{23}N_3O_8S$: C, 42.7; H, 5.9; N, 10.7; S, 8.2%). Recoveries better than 75% were obtained by adsorbing the product from the reaction mixture on Zeo-Karb 225 (H^+ form) resin, displacing it with dilute aq. NH_3 and, after evaporation of the solution, crystallizing the product from acetic acid and acetone. This procedure did not remove traces of oxidized glutathione and other ninhydrin-reacting compounds that were present.

METHODS

Phosphate buffers were prepared by adjusting the pH values of appropriate concentrations of NaH_2PO_4 with NaOH, and diluting the solution to the stated phosphate concentration. Tris buffers were similarly made by adding HCl to solutions of tris base.

Determination of glutathione. The 5,5'-dithiobis-(2-nitrobenzoic acid) reagent of Ellman (1959) was used under conditions similar to those described by Sedlak & Lindsay (1968). Enzymic reactions were stopped by the addition of 2.0 ml of reaction mixture to 0.5 ml of 10% (w/v) metaphosphoric acid. The mixture was centrifuged, and 0.5 ml samples of the supernatant, diluted if necessary so as to contain not more than 0.075 μ mol of thiol, were diluted with 4 ml of tris buffer (pH 8.2, 0.5 M) and 0.5 ml of 10 mM-5,5'-dithiobis-(2-nitrobenzoic acid). E_{412} was measured after 5 min, and corrected for a reagent blank.

Determination of the activity of glutathione S-crotonyl-N-acetylcysteamine transferase. The loss of unsaturated acyl thiol ester at 30°C was measured in 1 cm cuvettes at 263 nm in a Unicam SP.500 spectrophotometer. The test cuvette contained (concentrations in a final volume of 3 ml) phosphate buffer (pH 7.4, 0.05 M), S-crotonyl-N-acetylcysteamine (0.15 mM) and enzyme. The reaction was started by the addition of glutathione (0.5 mM), and the extinction was measured at 20 s intervals. The reference cuvette contained thiol ester to partly offset the extinction of the test cuvette. All reaction rates were corrected for the non-enzymic rate, which amounted to 0.0036 μ mol/min under the standard conditions. The initial rate was linear for at least 2 min and proportional to the concentration of enzyme with amounts of enzyme up to 0.06 U (1 unit of enzyme catalyses the loss of 1 μ mol of substrate per min). In measurements of the activity of the enzyme with other thiol esters the corresponding wavelength of maximum extinction was used (Table 2).

Tissue preparations. After homogenization at 0°C in 0.25 M-sucrose, subcellular fractions of rat liver and rat

kidney were prepared by centrifugation at 0°–2°C. The soluble fraction was that remaining in the supernatant after centrifugation at 100 000g for 1 h.

Chromatography and electrophoresis. Chromatograms were prepared on Whatman no. 1 and 3MM paper with butan-1-ol-acetic acid-water (12:3:5, by vol.) (solvent *A*) and butan-1-ol-pyridine-aq. 3M-NH₃ (1:1:1, by vol.) (solvent *B*). Electrophoretograms of amino acids were prepared on Whatman 3MM paper with aq. of pyridine (2%, v/v) and acetic acid (0.95%, v/v) at a field strength of 13 V/cm for 3 h.

Purification of glutathione *S*-alkene transferase. All steps were performed at 0–5°C. The supernatant fraction (130 ml) from 160 ml of a 20% (w/v) homogenate of rat liver was dialysed against four 2l portions of 2mM-tris buffer, pH 8.5, and the solution in the dialysis bag was then passed through a column of DEAE-cellulose (25 g) that had been equilibrated with the same buffer, and the column was eluted with buffer. The major band of protein-containing effluent (214 ml) was dialysed overnight against 10 litres of 2mM-phosphate buffer, pH 5.5, the solution in the dialysis bag was stirred with CM-cellulose (Na⁺ form, 3 g), and the pH of the mixture was adjusted to 5.5. The CM-cellulose was collected by centrifugation, transferred to a column with 40 ml of 2mM-phosphate buffer, pH 5.5, and washed in the column with a further 60 ml of buffer. The column was washed with 120 ml of 2mM-phosphate buffer, pH 5.5, containing 0.1M-NaCl and then with 2mM-phosphate buffer, pH 5.5, containing 0.2M-NaCl. Each eluate fraction (20 ml) was adjusted to pH 5.5 as it was collected, and fractions containing enzymic activity were combined (160 ml) and stored at –25°C. Portions (40 ml) were further purified by gradient elution on CM-cellulose. A column containing 50 g of ion-exchange material was equilibrated with phosphate buffer (pH 6.0, 0.05M). The enzyme solution was equilibrated with the same buffer by dialysis, applied to the column, and eluted with buffer delivered from apparatus similar to that described by Kellie & Wade (1957) for producing linear gradients. Initially the reservoir contained 500 ml of 0.086M-NaOH and the mixing vessel 500 ml of 0.05M-phosphate buffer, pH 6.0. Effluent fractions of 50 ml were collected, and two bands of activity were detected. The first and minor quantity was in fractions 4 to 7 inclusive and the second was in fractions 9, 10 and 11. When the

experiment was repeated and the second band was collected in smaller fractions (5 ml), certain fractions appeared with a tenfold higher specific activity than the mean value for the enzyme in this band, but the exact position of these fractions within the band could not always be reproduced. Fractions were stored at –25°C. Details of the purification are given in Table 3. The enzyme was conveniently concentrated by dialysing it against phosphate buffer (1 mM, pH 7) and then freeze-drying it.

Stoichiometry of the reaction. The chemistry of the reaction was demonstrated by measuring the stoichiometry of the loss of ester and glutathione and the formation of product [measured as *S*-(2-carboxy-1-methylethyl)cysteine after hydrolysis]. A reaction mixture in 0.05M-phosphate buffer, pH 7.4, containing both substrates at 0.15M was incubated at 30°C. Samples (2 ml) were removed at 5 min intervals, the E_{263} was measured to determine the ester concentration, and then 0.5 ml of 10% metaphosphoric acid was immediately added to stop the reaction before the glutathione concentration was determined. At 30 min after starting the reaction conc. HCl (3 ml) was added to the reaction mixture (15 ml), and the solution was heated on a boiling-water bath for 4 h and then evaporated to dryness at 45°C. Acidic amino acids were isolated from the reaction product by using ion-exchange resins as described for the preparation of carboxyalkylcysteines, and then dissolved in 2 ml of 2M-acetic acid. Chromatography and electrophoresis of this solution revealed the presence of glutamic acid, aspartic acid and a compound identical with *S*-(2-carboxy-1-methylethyl)cysteine. The quantity of the latter was determined by chromatography of accurately measured volumes (200 μ l) of the solution and comparing the colour produced in the ninhydrin reaction with that formed by known quantities of the authentic amino acid.

RESULTS AND DISCUSSION

The glutathione-dependent loss of *S*-crotonyl-*N*-acetylsteamine was catalysed to a significant extent only by the soluble fractions of rat liver and kidney, which were equally active on the basis of wet wt. of tissue. Freshly prepared and washed

Table 3. *Purification of glutathione S-crotonyl-N-acetylcysteamine transferase*

Details of methods and volumes are given in the text. Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951) with bovine serum albumin as standard.

Fraction	Enzyme concentration (U/ml)	Protein concentration (mg/ml)	Specific activity (U/mg)	Recovery (%)
Supernatant	1.17	20.2	0.058	(100)
Effluent from DEAE-cellulose	0.70	6.44	0.109	98
Effluent from first CM-cellulose column	0.75	1.33	0.564	78
Effluent from second CM-cellulose column				
i) fractions 9, 10 and 11	0.072	0.080	0.90	28
ii) fractions of 5 ml	0.154	0.016	9.62	2

Table 4. *Stoichiometry of the enzyme-catalysed reaction between glutathione and S-crotonyl- and S-methylcrotonyl-N-acetylcysteamine*

The complete reaction mixture contained substrates at a concentration of 0.15 mM. About 0.03 U of enzyme/ml was used for the crotonyl ester and 0.3 U/ml for the methylcrotonyl esters. In control experiments one of the reaction components was omitted. To measure the concentration of methylcrotonyl esters the sample of reaction mixture was diluted with an equal volume of water immediately before measurement of the extinction. Carboxyalkylcysteines were detected on chromatograms with ninhydrin. For details see the text.

Acyl group	System ...	Time (min)	Decrease in ester concentration (μmol)			Decrease in glutathione concentration (μmol)			Carboxyalkylcysteine formed			
			Complete	Lacking		Complete	Lacking		μmol	R_F in solvent		Distance moved on electrophoresis (cm)
				Enzyme	Glutathione		Enzyme	Ester		A	B	
Crotonyl	5	46	4	1	48	4	1					
	10	64	6	0	65	5	2					
	15	76	9	1	79	10	3					
	20	88	10	2	89	10	1					
	22	95	13	3	99	14	4					
	30	99	15	2	103	16	5	105	0.43 (0.45)*	0.36 (0.37)*	12.0 (12.0)*	
2-Methylcrotonyl	30	69	3	1	70	0	1	72	0.53 (0.54)†	0.45 (0.44)†	4.0 (13.8)†	
3-Methylcrotonyl	30	55	1	0	55	2	2	65	0.52 (0.54)‡	0.44 (0.46)‡	13.8 (13.9)‡	

* Values for authentic *S*-(2-carboxy-1-methylethyl)cysteine.

† Values for authentic *S*-(2-carboxy-1-methylpropyl)cysteine.

‡ Values for authentic *S*-(1-carboxy-2-methylpropyl)cysteine.

nuclear, mitochondrial and microsomal fractions had no significant activity. The enzyme was purified 160-fold from the soluble fraction of rat liver (Table 3).

The results of experiments to determine the stoichiometry of the reaction are given in Table 4, and show a close correlation between the disappearance of ester and glutathione during the course of the reaction, and also good agreement between the amount of substrates lost and the amount of *S*-(2-carboxy-1-methylethyl)cysteine found after hydrolysis. Neither substrate disappeared when incubated with the enzyme alone and under these conditions no *S*-(2-carboxy-1-methylethyl)cysteine could be detected after hydrolysis. The control reaction from which only enzyme had been omitted probably gave more *S*-(2-carboxy-1-methylethyl)cysteine than could have been formed up to the stage at which hydrochloric acid was added to the reaction mixture, but this quantity was too small to measure accurately. This was probably because of a continuing addition of cysteine or cysteine peptides to the ester or to crotonic acid during the hydrolysis, an additional production that would occur to a lesser extent in the mixture containing enzyme because of the much larger consumption of substrates that had occurred in the previous step. When, after 30 min an equal volume of a 10% (w/v) homogenate of rat kidney in 0.05M-phosphate buffer, pH 7.4, was added to the complete reaction mixture and the incubation was continued for a further 2 h, acidic amino acids that were isolated from the reaction mixture without acid hydrolysis contained a compound chromatographically and electrophoretically identical with *S*-(2-carboxy-1-methylethyl)-cysteine.

The non-enzymic reaction of glutathione with 2,3-unsaturated acyl thiol esters, that had been noted by Stern, del Campillo & Raw (1956), is not a simple one. Chromatography of the reaction products formed from *S*-crotonyl-*N*-acetylcysteamine allows at least three reaction products to be distinguished, and this may account for the observation that neither the enzymic nor the non-enzymic reactions, measured with equimolar quantities of substrates or with an excess of glutathione, proceed to completion.

The concentrations of substrates used in the standard assay were less than saturating to limit the non-enzymic reaction rate to 0.0036 $\mu\text{mol/min}$. This rate increases very rapidly with increasing pH values, and for this reason we were not able to measure the optimum pH for the enzymic reaction accurately. It is probably in the region of pH 8 to pH 9.

The kinetic parameters K_m and V_{max} for the reaction of glutathione with *S*-crotonyl-*N*-acetyl-

Table 5. K_m and V_{max} values for the enzyme-catalysed reaction of glutathione with a number of 2,3-unsaturated acyl thiol esters at 30°C and pH 7.4

For details of methods see the text. The K_m values for the esters were determined with 0.5 mM-glutathione, and that for glutathione with 0.15 mM-*S*-crotonyl-*N*-acetylcysteamine. These concentrations are not saturating. The parameters listed were determined for straight lines fitted to Lineweaver-Burk plots by the method of least squares.

Substrate	K_m (mM)	V_{max} .
<i>S</i> -Crotonyl- <i>N</i> -acetylcysteamine	0.10	100
<i>S</i> -(3-Methylcrotonyl)- <i>N</i> -acetylcysteamine	0.074	2.8
<i>S</i> -(2-Methylcrotonyl)- <i>N</i> -acetylcysteamine	0.080	15.0
<i>S</i> -Crotonylpantetheine	0.052	25.4
<i>S</i> -Crotonyl- <i>N</i> -acetyl-L-cysteine	0.26	50.5
<i>S</i> -Crotonyl- <i>NN</i> -dimethylcysteamine	0.067	49.3
Glutathione	0.082	

cysteamine and other thiol esters are given in Table 5. These values were measured under the conditions of the standard assay method, except that the concentration of one substrate was varied in turn. It should be noted, however, that the concentration of the fixed substrate used in these measurements was less than saturating, in order to limit the non-enzymic reaction. *S*-Crotonyl-*N*-acetylcysteamine was the most active substrate, *S*-crotonylpantetheine was somewhat less active, and the substitution of the crotonyl residue with a methyl group at either the 2 or the 3 position considerably decreased V_{max} , though not the K_m . The reaction of glutathione with two other *S*-crotonyl esters was also catalysed, but the results for the *NN*-dimethylcysteamine are subject to some uncertainty because of the very rapid non-enzymic reaction. *S*-(2-Carboxy-1-methylethyl)glutathione and oxidized glutathione behaved as competitive inhibitors when the concentration of glutathione was varied (Fig. 1). The enzyme did not catalyse a reaction between glutathione and ethyl crotonate, crotonic acid, ethyl maleate or ethyl fumarate. In the enzymic reaction between glutathione and *S*-crotonyl-*N*-acetylcysteamine, neither L-cysteine nor *N*-acetyl-L-cysteine could replace glutathione.

The broad specificity of the enzyme suggests that it could be responsible for the reaction leading to the production of certain *S*-carboxyalkylcysteines. Those that have been found in animal urine and that might be formed as a consequence of similar reactions are given in Table 1. There are unresolved problems, however, associated with this suggestion. The metabolic intermediates listed in Table 1 are CoA derivatives, and though they have not been used in the present studies they would probably be consumed more rapidly than in those examined here in normal degradative pathways.

Fig. 1. Lineweaver-Burk plots for the enzyme catalysed reaction of glutathione with *S*-crotonyl-*N*-acetylcysteamine. The lines were fitted to the results by the method of least squares. ○, No inhibitor present; □, 0.5 mm-oxidized glutathione present; Δ, 0.01 mm-*S*-(2-carboxy-1-methyl-ethyl)glutathione present.

This is particularly true because of the compartmentation of the CoA derivatives within the mitochondria, whereas the enzyme examined in the present studies occurs in the cell sap. The circumstances under which 2,3-unsaturated acyl-CoA derivatives or their partial hydrolysis products have access to the cell sap are not known, but may have significance for the increased excretion of carboxyalkylcysteines in pathological conditions.

There is also an uncertainty about the chemistry of the reaction catalysed by the enzyme. It has been assumed that the S of glutathione adds to the C atom of the double bond remote from the ester group, as would be expected on chemical grounds, but it is probable that the conditions of chromatography and electrophoresis used in the present studies would not distinguish the possible structural isomers. It cannot, therefore, be assumed (as we have in Table 4) that the amino acid derived from *S*-(3-methylcrotonyl)-*N*-acetylcysteamine was *S*-(1-carboxy-2-methylpropyl) cysteine. It might have been *S*-(2-carboxy-1,1-dimethylethyl)cysteine and the only reason for assuming that it is the former amino acid is that it is the one formed *in*

vivo. If the amino acids listed in Table 1 are produced via the reaction described here, the reaction involving 3-methylcrotonyl esters must have unusual directional properties. It may be that binding of the ester substrate is such as to allow the presentation of the least hindered C atom of the double bond to the initial attack of glutathione.

Finally it should be mentioned that reactions of the type discussed above cannot account for the production of all the carboxyalkylcysteines [e.g. *S*-(1,2-dicarboxyethyl)cysteine] and some that could be produced by these reactions may be derived from alternative sources. Thus a plausible route to the formation of *S*-(2-carboxyethyl)-cysteine is the deamination and oxidative decarboxylation of the C₄ unit of cystathionine.

The authors thank the Wellington Medical Research Foundation for support, and E.A.B. thanks the Science Research Council for a Research Fellowship.

REFERENCES

- Boyland, E. & Chasseaud, L. F. (1967). *Biochem. J.* **104**, 95.
- Boyland, E. & Chasseaud, L. F. (1968). *Biochem. J.* **109**, 651.
- Ellman, G. L. (1959). *Archs Biochem. Biophys.* **82**, 70.
- Fowden, L. (1964). *A. Rev. Biochem.* **33**, 173.
- Fukutome, K. (1961). *J. Biochem., Tokyo*, **49**, 444.
- Fukutome, K. (1962a). *Acta Med. Okayama*, **16**, 241.
- Fukutome, K. (1962b). *Acta Med. Okayama*, **16**, 247.
- Kellie, A. E. & Wade, A. P. (1957). *Biochem. J.* **66**, 196.
- Kodama, H. (1968). *Biochim. biophys. Acta*, **165**, 432.
- Kuwaki, T., Ohmori, S. & Mizuhara, S. (1963). *Biochim. biophys. Acta*, **78**, 553.
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). *J. biol. Chem.* **193**, 265.
- Lynen, F. (1969). In *Methods in Enzymology*, vol. 14, p. 17. Ed. by Lowenstein, J. M. New York: Academic Press Inc.
- Ohmori, S., Shinomura, T., Azumi, T. & Mizuhara, S. (1965). *Biochem. Z.* **343**, 9.
- Oomori, S. & Mizuhara, S. (1962). *Archs Biochem. Biophys.* **96**, 179.
- Sedlak, J. & Lindsay, R. H. (1968). *Analyt. Biochem.* **25**, 192.
- Stern, J. R., del Campillo, A. & Raw, I. (1956). *J. biol. Chem.* **218**, 971.
- Suzuki, T., Sugii, M. & Kakimoto, T. (1962). *Chem. pharm. Bull., Tokyo*, **10**, 328; cited in *Chem. Abstr.* (1962) **57**, 11561.