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Elevation of Endogenous Nucleophiles in Rat Lung by Cysteine and Glutathione Esters *In Vitro*

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ABSTRACT. In this study, we have compared the uptake of L-cysteine (L-CySH), D-cysteine (D-CySH), L-cysteine isopropyl ester (L-CIPE) and D-cysteine isopropyl ester (D-CIPE) in rat lung slices and tracheal sections and determined the effectiveness of glutathione (GSH), GSH isopropyl monoester, GSH isopropyl diester, γ-glutamylcysteine (γ-glu-cys) isopropyl monoester and γ-glu-cys isopropyl diester to elevate and prolong intracellular GSH concentrations in rat lung slices. Lung slices were incubated with 1.0 mM of thiol and the concentrations determined intracellularly and extracellularly with time. Slices incubated with GSH, GSH isopropyl diester and γ-glu-cys isopropyl diester had cellular GSH concentrations increased by up to 60%, 95% and 58%, respectively, whereas GSH isopropyl monoester and γ-glu-cys isopropyl monoester did not increase the intracellular GSH concentration. Extracellularly, the GSH concentration had decreased by 15%, GSH isopropyl diester by 27%, γ-glu-cys isopropyl diester by 66% and both isopropyl monoesters by over 90% at 120 min. Lung slices and tracheal sections incubated with L- or D-CySH at 37° had increased cellular concentrations of L- and D-CySH which ranged between 0.88–1.25 nmol mg^{-1} and 1.35–2.25 nmol mg^{-1}, respectively. Reducing the incubation temperature to 4° had little effect on the accumulation of D-CySH; however, L-CySH concentrations increased progressively in the trachea and lung to reach 2.73 and 2.63 nmol mg^{-1} at 90 min, respectively. Lung slices incubated with L- or D-CIPE had increased L- or D-CySH concentrations up to a max of 13.7 and 11.1 nmol mg^{-1} and tracheal sections up to a max of 5.56 and 11.09 nmol mg^{-1}. In the lung slice medium, L- and D-CIPE levels had decreased by 75.2% and 74.0% at 90 min, respectively, and from the tracheal section medium, L- and D-CIPE concentrations had decreased by 66.7% and 32.7%, respectively. Preincubation of lung slices and tracheal sections with the carboxylesterase inhibitor, bis (*p*-nitrophenyl) phosphate (BNPP), almost completely prevented the disappearance of L- and D-CIPE extracellularly and greatly reduced the appearance of cellular L- and D-CySH. GSH, GSH isopropyl diester and γ-glu-cys isopropyl diester elevated and prolonged GSH concentrations in rat lung slices, but GSH isopropyl monoester and γ-glu-cys isopropyl monoester did not increase GSH levels. The uptake of L-CySH, but not D-CySH, is temperature sensitive in rat lung slices and tracheal sections and carboxylesterases appear to have a major influence on the uptake and metabolism of L- and D-CIPE by rat lung slices and tracheal sections. BIOCHEM PHARMACOL **55**;10:1573–1584, 1998. Crown Copyright © 1998. Published by Elsevier Science Inc.

KEY WORDS. lung; trachea; cysteine; glutathione; cysteine esters; glutathione esters

Most cell types are unable to import intact GSH† and instead rely on its catalysis by γ-glutamyl transpeptidase and subsequent uptake of the constituent amino acids into the cell which are resynthesised in two reactions catalysed by γ-glu-cys synthetase and GSH synthetase [1]. An increase in the cellular concentration of GSH exerts a negative feedback on γ-glu-cys synthetase, which is the rate limiting step. Certain cell types, such as rat alveolar type II cells, rat small-intestinal epithelial cells and rat kidney cells, can transport intact GSH by a Na^+ dependent system and in rat hepatocytes a GSH transporter can operate bidirectionally under certain conditions [2–5]. All cell types have the capacity to export GSH, either in the reduced or oxidised form, and, in particular, the extracellular mileau of the lung is rich in GSH at the alveolar epithelial surface [1].

Endogenous nucleophiles can offer protection to the cell from a toxic insult by electrophilic compounds, either directly or by enzyme mediated reactions [1]. In situations where there may be a risk from exposure to toxic compounds, increasing the intracellular levels of nucleophiles prior to the exposure may reduce the toxic insult to the cell. In rats, the CySH concentration was increased in the bronchio-alveolar lavage fluid and in the lung following i.p. administration of esters of CySH. CySH esters also prevented lethality in rats that were exposed to PFIB, a hydrophobic gas that causes cellular damage to the lower

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† *Abbreviations:* BNPP, bis (p-nitrophenyl) phosphate; CIPE, cysteine isopropyl ester; CySH, cysteine; γ-glu-cys, γ-glutamylcysteine; GSH, glutathione; mBBr, monobromobimane; PFIB, perfluoroisobutene.

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respiratory tract, leading to pulmonary oedema, and also protected lung slices from sulphur mustard poisoning [6–9].

By using a lung slice model, we previously determined the structure-activity relationships and mechanism(s) of CySH elevation in the lung by CySH esters [10]. The esters were very effective at raising intracellular CySH levels; however, export of CySH from the lung slice was very efficient so that the esters could not be considered for use *in vivo* as a pretreatment regime. Consequently, a pretreatment is required that will not only increase the intracellular concentrations of nucleophilic thiols, but also increase their residence time in the cell so as to increase the duration of protection. In this study, we have determined the effectiveness of four thiol esters, GSH isopropyl monoester, GSH isopropyl diester, γ-glu-cys isopropyl monoester and γ-glu-cys isopropyl diester to elevate and prolong intracellular GSH concentrations in rat lung slices.

N-acetyl-L-CySH and *N*-acetyl-D-CySH isomers prevented an increase in lung oedema to the same extent when administered intravenously in a hyperoxic-induced lung damage model in the rat [11]. In this study, we used a lung slice and tracheal section model to determine the uptake and metabolism of L- and D-isomers of CySH and CIPE. If intracellular D-CySH, produced by hydrolysis, is exported at a different rate from the L-form the residence time in the cell may be increased. The formation of the thiolate anion is important to the function of the molecule as a nucleophile and the isomers were studied because they ionize to the same degree, but may not be hydrolysed at the same rates [12]. BNPP, an inhibitor of carboxylesterases, was used to determine the characteristics of the enzymes involved in the metabolism of the L- and D-CySH esters in the lung and trachea [13].

MATERIALS AND METHODS

Animals

Female Porton Wistar rats, 180–220 g, were used in this study.

Chemicals

GSH (reduced), L-CySH, D-CySH and BNPP were purchased from Sigma. L-GSH isopropyl monoester, L-GSH isopropyl diester, γ-glu-cys isopropyl monoester, γ-glu-cys isopropyl diester, L-CIPE, D-CIPE and mBBr were synthesised at CBD, Porton Down. Pentobarbitone (Sagatal) was purchased from May and Baker Ltd, Dagenham, England.

SYNTHESIS OF THE THIOL ESTERS

Cysteine Esters

CIPE was synthesised as described previously by Lailey *et al.* [7].

GSH Isopropyl Monoester

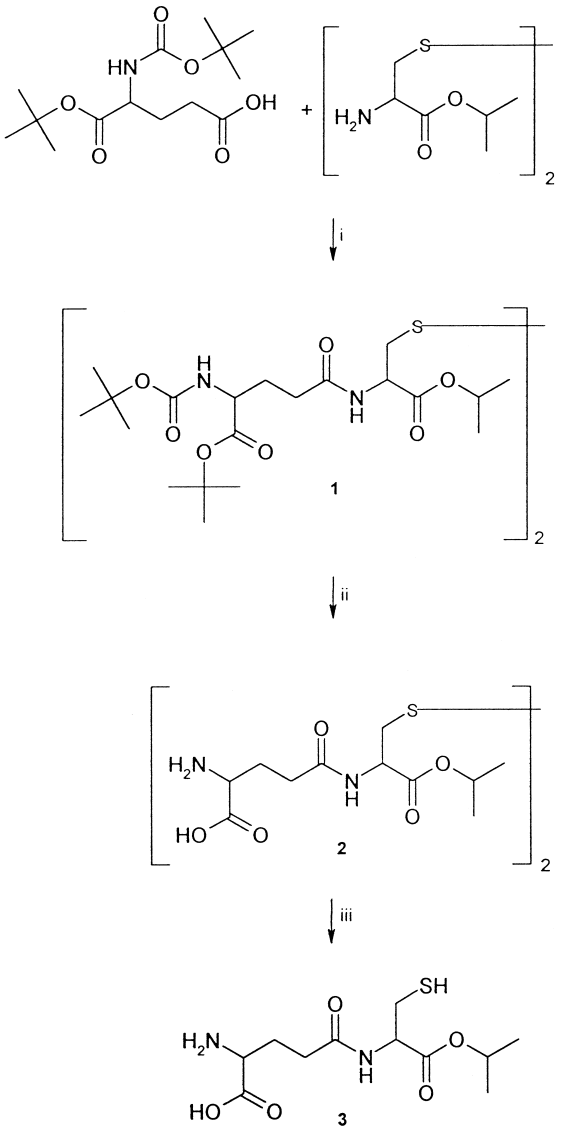
GSH isopropyl monoester was synthesised as described previously by Anderson *et al.* by using method B with sulphuric acid [14].

GSH Isopropyl Diester

Concentrated sulphuric acid (12.5 mL) was added dropwise to a stirred solution of GSH (25.0 g, 81.4 mmol) under argon and the mixture was stored at room temperature for 3 days. HPLC analysis (conditions described below) showed the presence of four products that were identified as both monoesters, the diester and a cyclised product. The mixture was concentrated under reduced pressure, the pH was adjusted to 7.5 by the addition of 10% sodium bicarbonate and it was extracted with dichloromethane (3 × 200 mL). The extracts were combined, dried and concentrated under reduced pressure to produce the required ester slightly contaminated with oxidised material. The impure ester was dissolved in a minimum amount of water, the pH was adjusted to 7.0 by the addition of 10% hydrochloric acid and excess dithiothreitol was added. After 17 hr, analysis by HPLC showed the reaction to be complete. The mixture was acidified to pH 2, extracted with ether (3 × 50 mL) and the extracts were discarded. The aqueous phase was then adjusted to pH 7–8, extracted with ether (3 × 50 mL) and the combined extracts were dried over anhydrous magnesium sulphate. Concentration at reduced pressure gave the title compound (5.1 g, 15.8%).

γ-Glu-Cys Isopropyl Monoester

N-α-*t*-Boc-L-glutamic acid α-*t*-butyl ester-cystine isopropyl monoester (Fig. 1, **1**): To a stirred solution of *N*-α-*t*-boc-L-glutamic acid (2 g, 6.7 mmol) in dry tetrahydrofuran (10 mL) at room temperature under argon, hydroxybenzotriazole (0.98 g, 7.26 mmol) was added, followed by dropwise addition of 1,3-dicyclohexylcarbodiimide (1.5 g, 7.27 mmol) in dry tetrahydrofuran (10 mL). After 60 min, diisopropyl cystine ester (1.05 g, 3.26 mmol) in dry tetrahydrofuran (10 mL) was added dropwise and the mixture stirred for a further 60 min. The reaction mixture was filtered and the filtrate concentrated at reduced pressure to produce a residual syrup (4 g). The syrup was dissolved in ethyl acetate (100 mL) and washed successively with 5% aqueous hydrochloric acid (50 mL), water (50 mL), 10% aqueous sodium hydrogen carbonate (50 mL) and water (50 mL), dried over anhydrous magnesium suphate and concentrated at reduced pressure to give the crude protected dipeptide (3.5 g). Purification by column chromatography on silica gel (column size 150 × 50 mm, eluting with ethyl acetate-petrol ether, 2:3, retention time of the fraction containing the product 60 min) gave the required protected dipeptide (2.3 g, 32%). In the NMR assignments, '●●'● = apparent coupling measured when system is second order, d = doublet, t = triplet and obs = obscured signal. ^{1}H δ



i hydroxybenzotriazole / 1,3-dicyclohexylcarbodiimide / tetrahydrofuran
ii trifluoroacetic acid / triethylsilane / dichloromethane
iii dithiothreitol / water

FIG. 1. Synthesis of L-γ-glu-cys isopropyl monoester. 1: *N*-α-*t*-Boc-L-glutamic acid α-*t*-butyl ester-cystine isopropyl monoester. 2: L-γ-glu-cystine isopropyl monoester. 3: L-γ-glu-cys isopropyl monoester.

($CDCl_3$) **1.25** (12H, d, J = 6.4 Hz, i-pr (CH_3), **1.40**, **1.44** (18H each, t-bu CH_3), **1.90**, **2.15** (2H each, m, CCH_2), **2.40** (4H, t, J = 6.0 Hz, $CH_2C=O$), **3.17** (4H, 'd', 'J' = 5.2 Hz, CH_2S), **4.17** (2H, br, glu-C*H*), **4.75** (2H, br dd, J = obs, cys-C*H*N), **5.05** (2H, septet, J = 6.4 Hz, C*H*O), **5.27** (2H, br, cys-N*H*), **6.83** (2H, br d, glu-N*H*).

L-γ-glu-cystine isopropyl monoester (Fig. 1, **2**): Trifluoroacetic acid (5 mL) and triethylsilane (2 mL) were added to a stirred solution of the dipeptide (4.3 g, 4.8 mmol) in dry dichloromethane (10 mL). After storing for 17 hr at room temperature, the mixture was concentrated at reduced pressure and the residue analysed by NMR. This showed the reaction to be incomplete. The residue was dissolved in dry dichloromethane (10 mL), and trifluoroacetic acid (5 mL) and triethylsilane (2 mL) were added. After 17 hr, the mixture was concentrated at reduced pressure to produce the pure deprotected peptide (4.1 g, 95%). ^{1}H δ (CD_3OD) **1.25** (12H, d, J = 6.4 Hz, CH_3), **2.20** (4H, m, CCH_2), **2.55** (4H, t, J = 7.0 Hz, $CH_2C=O$), **3.00** (4H, dd, J = 11.8, 8.8 Hz, CH_2S), **3.20** (4H, dd, J = 11.8, 4.9 Hz, CH_2S), **4.00** (2H, t, J = 7.8 Hz, glu-C*H*N), **4.67** (2H, dd, J = 8.8 and 4.9 Hz, cys-C*H*N), **5.05** (2H, septet, J = 6.4 Hz, C*H*O).

L-γ-glu-cys isopropyl monoester (Fig. 1, **3**): Dithiothreitol (1.01 g, 6.54 mmol) was added to a stirred solution of the oxidised dipeptide (4.1 g, 5.1 mmol) in water (30 mL). After storing at room temperature for one week, analysis by HPLC (conditions described below) showed the reaction to be complete, the product having a retention time of 22 min. The reaction mixture was extracted with dichloromethane (2 × 30 mL), and the extracts were analysed by HPLC and then were discarded. The aqueous phase was concentrated at reduced pressure to give a hygroscopic glass which was purified by preparative HPLC to give the title compound (0.65 g, 32%). ^{1}H δ (CD_3OD) **1.25** (6H, d, J = 6.2 *Hz*, CH_3), **2.20** (2H, m, CCH_2), **2.57** (2H, t, J = 7.3 Hz, $CH_2C=O$), **2.95** (1H, dd, J = 13.9 and 4.5 Hz, CH_2S), **2.85** (1H, dd, J = 13.8 and 6.8 Hz, CH_2S), **4.00** (1H, t, J = 7.3 Hz, glu-C*H*N), **4.56** (1H, dd, J = 4.7 and 6.8 Hz, cys-C*H*), **5.03** (1H, septet, J = 6.1 Hz, C*H*O).

γ-Glu-Cys Isopropyl Diester

N-α-Boc-L-glutamic acid α-isopropyl γ-benzyl ester: A solution of *N*-α-*t*-boc-L-glutamic acid-γ-benzyl ester (10 g, 29.6 mmol), 1,8-diazabicycloundecene (4.5 g, 32 mmol), 2-bromopropane (4 g, 32 mmol) and a catalytic amount of tetrabutylammonium bromide (100 mg) in dry tetrahydrofuran (150 mL) were heated under reflux. After 9 hr, the solvent was removed under reduced pressure and the residue was dissolved in dichloromethane (150 mL). The organic layer was washed with water (50 mL), hydrochloric acid (1 M, 50 mL), and a 10% aqueous solution of sodium hydrogen carbonate (50 mL), dried over anhydrous magnesium sulphate and concentrated at reduced pressure to give pure product (11 g, 98%). ^{1}H δ ($CDCl_3$): **7.30** (5H, m, Ar), **5.1** (2H, s, $ArCH_2$), **5.05** (1H, br, N*H*), **5.05** (5H, septet, J = 5 Hz, C*H*O), **4.25** (1H, br m, C*H*NH), **2.40** (2H, complex m, $CH_2CH_2C=O$), **2.15–1.95** (1H, each br m, $CH_2CH_2C=O$), **1.41** (9H, s, Me_3), **1.23–1.22** (3H, each d, J = 5 Hz Me_2).

N-α-*t*-Boc-L-glutamic acid α-isopropyl ester: The boc protected benzyl ester (7 g, 18.5 mmol) was dissolved in tetrahydrofuran (150 mL). A catalytic amount of 10% palladium on charcoal was added and the mixture was hydrogenated until hydrogen uptake ceased. The suspension was filtered and the filtrate was concentrated at reduced pressure to give pure product (6.2 g, 98%). ^{1}H δ

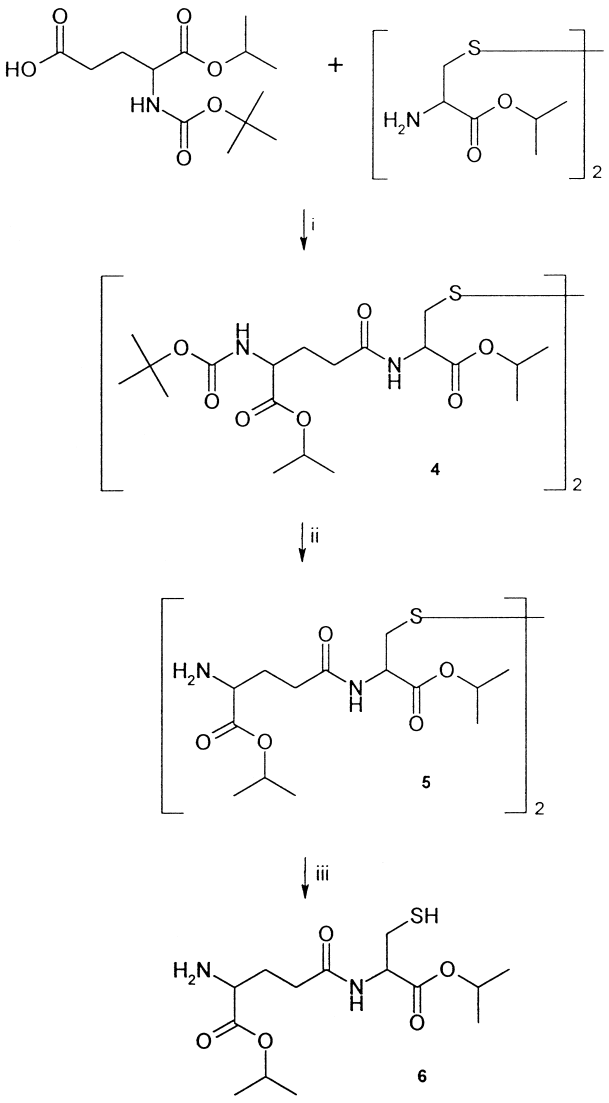


FIG. 2. Synthesis of L-γ-glu-cys isopropyl diester. 4: *N*-α-*t*-boc-L-glu-α-isopropyl ester-cystine isopropyl ester. 5: L-γ-glu-cystine isopropyl diester. 6: L-γ-glu-cys isopropyl diester.

($CDCl_3$): **5.20** (1H, br, N*H*), **5.02** (1H, septet, C*H*O), 4.20 (1H, br, C*H*N), **2.50** (2H, m, $CH_2C=O$), **2.00** (2H, br, CH_2CH_2), **1.41** (9H, s, Me_3), **1.22–1.24** (3H each, 2 d, Me_2). ^{13}C δ ($CDCl_3$): **177.5**, **177.1** (C = O, CO_2H and CO_2), **155.5** (NC = O), **80.1** ($OCMe_3$), **69.4** (*C*HO), **67.9** (CHN), **30.1** ($CH_2C=O$), **25.6** (CH_2CH), **30.1**, **28.4** (Me_2), **28.3** (Me_2).

N-α-*t*-Boc-L-glu-α-isopropyl ester-cystine isopropyl ester (Fig. 2, **4**): To a stirred solution of *N*-α-*t*-boc-L-glutamic acid isopropyl ester (1.75 g, 6.1 mmol) in dry tetrahydrofuran (10 mL) at room temperature under argon, hydroxybenzotriazole (0.9 g, 6.7 mmol) was added, followed by dropwise addition of 1,3-dicyclohexylcarbodiimide (1.38 g, 6.7 mmol) in dry tetrahydrofuran (5 mL). After 60 min, the cystine ester (0.98 g, 3.04 mmol) in dry tetrahydrofuran (10 mL) was added dropwise and the resultant mixture was stirred for an additional 60 min. The reaction mixture was filtered and the filtrate was washed, in turn, with 5% aqueous hydrochloric acid (50 mL), water (50 mL), 10% aqueous sodium hydrogen carbonate (50 mL) and water (50 mL), dried over anhydrous magnesium sulphate and concentrated at reduced pressure to give the crude dipeptide (2.5 g). Purification of the residue by column chromatography on silica gel (column size 150 × 50 mm) eluting with ethyl acetate-petrol ether (2:3, retention time of the fraction containing the product was 60 min), produced the pure dipeptide (1.4 g, 32%). ^1H δ ($CDCl_3$) **1.25** (24H, m, iprCH_3), **1.45** (18H, s, *t*-buCH_3), **2.00** (4H, m, CCH_2), **2.40** (4H, br t, 7.2 Hz, CH_2C = O), **3.30** (4H, br 'd', CH_2S), **4.20** (2H, br, glu-C*H*), **4.65** (2H, m, cys-C*H*), **5.00** (4H, m, C*H*O), **5.30** (2H, br, cys-N*H*), **6.80** (2H, br, glu-N*H*).

L-γ-glu-cystine isopropyl diester (Fig. 2, **5**): Trifluoroacetic acid (5 mL) and triethylsilane (2 mL) were added to a stirred solution of the dipeptide (3.2 g, 3.7 mmol) in dry dichloromethane (10 mL). After 17 hr, the mixture was concentrated at reduced pressure to produce the pure deprotected peptide (3.2 g, 97%). ^1H δ ($CDCl_3$) **1.25** (12H, 2 d's, *J* = 7.0 Hz, CH_3), **2.20**, **2.35** (2H each, m, CCH_2), **2.65** (2H, br t, '*J*' = 3 Hz, CH_2C = O), **3.05** (2H, dd, *J* = 5.2 and 14.1 Hz, CH_2S), **3.20** (2H, dd, *J* = 7.1 and 14.1 Hz, CH_2S), **4.10** (2H, m, glu-C*H*N), **4.70** (2H, dd, 7.8 and 5.2 Hz, cys-C*H*), **5.05** (4H, 2 septets, *J* = 7.0 Hz, C*H*O), **7.30** (2H, br d, *J* = 7.5 Hz, N*H*).

L-γ-glu-cys isopropyl diester (Fig. 2, **6**): The oxidised dipeptide (3.0 g, 3.4 mmol) was dissolved in water (50 mL), the pH was adjusted to 8.0 with 10% aqueous sodium hydrogen carbonate, and dithiothreitol (0.63 g, 4.1 mmol) was added. After 3 hr, the mixture was analysed by TLC (using 9:1 chloroform saturated with ammonia-methanol) and found to be complete. The mixture was concentrated at reduced pressure. The residual oil was taken up in hexane and afforded a small amount of the required product as a white crystalline solid (0.2 g, 7%). The mother liquid was concentrated under reduced pressure and analysis of the residue by TLC now showed that some product had oxidised back to the disulphide starting material. No attempts were made to optimise the process. ^1H δ ($CDCl_3$) **1.25** (12H, 2 d's, *J* = 7.0 Hz, CH_3), **2.20**, **2.35** (2H each, m, CCH_2), **2.65** (2H, br t, '*J*' = 3 Hz, CH_2C = O), **3.05** (2H, dd, *J* = 5.2 and 14.1 Hz, CH_2S), **3.20** (2H, dd, *J* = 7.1 and 14.1 Hz, CH_2S), **4.10** (2H, m, glu-C*H*N), **4.70** (2H, dd, 7.8 and 5.2 Hz, cys-C*H*), **5.05** (4H, 2 septets, *J* = 7.0 Hz, C*H*O), **7.30** (2H, br d, *J* = 7.5 Hz, N*H*).

CHROMATOGRAPHY CONDITIONS

Analytical and preparative separations were carried out using a Gilson HPLC system (Anachem, Luton, UK) comprising two model 303 pumps, a model 117 variable wavelength UV detector, a model 202 autoinjector, a

model 803 manometric module and a model 811 dynamic mixer. It was controlled by an IBM PS/2 model 70 386 computer using Gilson 715 HPLC and System Manager software (version 1.01). A Rheodyne 7010 injector was used. HPLC grade acetonitrile (Romil, Loughborough, UK) and water (18 MΩ obtained in-house using an Elgastat UHQ II water purifier) were used for chromatography. The two solvent systems contained 0.1% v/v trifluoroacetic acid. For the initial analysis a Vydac protein and peptide C18 column was used, size 4.6 × 250 mm, particle size 10 μ and pore size 300 Å. A matching preparative column size 22 × 250 mm, particle size 10–15 μ and pore size 300 Å, was used for purification. The preparative scale sample loadings were 250 mg. The mobile phase comprised 0.1% trifluoroacetic acid in water (solvent A) and 0.1% trifluoroacetic acid in acetonitrile (solvent B).

Separation Gradient for GSH Isopropyl Diester

The solvent gradient comprised 0–2 min 5% solvent B, 2–25 min a linear gradient of 5–40% solvent B, 25–29 min a linear gradient of 40%–90% solvent B, 29–32 min 90% solvent B, 32–35 min a linear gradient of 90–5% solvent B and 35–40 min 5% solvent B. The flow rate was 1.0 mL min^{-1} and the eluate was monitored using a UV detector at wavelengths 214 and 225 nm.

Separation Gradient for L-γ-glu-cys Isopropyl Monoester and Diester

The solvent gradient was as follows: 0–7 min 5% solvent B, 7–38 min a linear gradient of 5–40% solvent B, 38–41 min a linear gradient of 40–90% solvent B, 41–46 min 90% solvent B and 46–49 min a linear gradient of 90–5% solvent B. The flow rate was 1 mL min^{-1} for analytical runs and 21.4 mL min^{-1} for preparative scale chromatography. The eluate was monitored using a UV detector at wavelengths 214 and 225 nm. TLC was performed on MK6F silica gel (60 Å) plates (Whatman) with detection by UV (254 nm) and iodine vapour.

PREPARATION OF THE TISSUES

Lung Slices

Rats were humanely killed with pentobarbitone (300 mg kg^{-1}) and the lungs perfused with 0.9% saline until they were blanched in appearance. The largest lobe of the lung was sliced to a thickness of 0.5 mm by using a McIlwain tissue slicer (Mickle Laboratory Engineering, Gomsholl Surrey, U.K.) and washed thoroughly with Krebs physiological salt solution (118 mM of NaCl, 1.0 mM of $MgSO_4 \cdot 7H_2O$, 1.2 mM of KH_2PO_4, 4.8 mM of KCl, 25 mM of $NaHCO_3$, 2.6 mM of $CaCl_2$ and 11.1 mM of glucose) at pH 7.40 before use. Two slices of approximately equal size and mass (12–14 mg each) were used for each incubation. Triplicate incubations were carried out at each time point for each animal and three animals were used for each compound.

Tracheal Sections

Rats were humanely killed with pentobarbitone (300 mg kg^{-1}) and the trachea was removed and washed in Krebs. Connective tissue was carefully and thoroughly removed and the trachea was cut in half longitudinally and each half was cut into three equal sections giving a total of six approximately square sections. Two sections of approximately equal size and mass (6–8 mg each) were used for each incubation. Triplicate incubations were carried out at each time point for each animal and three animals were used for each compound.

Incubation of the Lung Slices and Tracheal Sections with Selected Thiols

Two lung slices or two tracheal sections were incubated in 5.0 mL of Krebs in the presence of selected thiol (1 mM) and were gently agitated in a shaking water bath at 50 strokes min^{-1} at 37° or 4°. At 0, 15, 30, 60, 90 and 120 min, both of the lung slices or both of the tracheal sections were removed and homogenised in 900 μL of mBBr (0.5 mg mL^{-1} made up in 5 mM of *N*-ethyl morpholine buffer, pH 8.0). At the same time 50-μL samples of the incubation medium were removed and derivatised in 850 μL of mBBr. After complete derivatisation, 100 μL of methane sulphonic acid (10% v/v) was added and then these were centrifuged at 13,000 r.p.m. for 5 min (MSE Micro-Centaur). Samples of 10 μL were used for HPLC analysis.

Incubation with BNPP

Half of the slices or sections were incubated in Krebs and half in 1.0 mM of BNPP for 30 min at 37°. The slices or sections were then washed and incubated at 37° in 1.0 mM of L-CIPE or D-CIPE as described above.

HPLC Analysis

The method is one modified from Kosower *et al.* [15]. The equipment used: Two Waters HPLC pumps, models 6000A and M510, Waters Automated Grant Controller, Perkin Elmer LS5 Luminescence Spectrometer, a Waters 712 WISP automatic injector and a Hewlett Packard 3393A Integrator. Column: HPLC Technology Hypersil 3ODS 150 mm × 4.6 mm with an Upchurch guard column packed with Perisorb RP18. L-CySH, D-CySH, L-GSH, L-CIPE, D-CIPE, L-GSH isopropyl monoester, γ-glu-cys isopropyl monoester, L-GSH isopropyl diester and γ-glu-cys isopropyl diester were separated using a gradient controlled program which gave retention times of 2.80, 2.80, 3.85, 9.60, 9.60, 10.82, 10.99, 12.53 and 12.60 min, respectively. It is important to note that this method cannot separate the L- and D-isomers of CySH and CIPE and so these have the

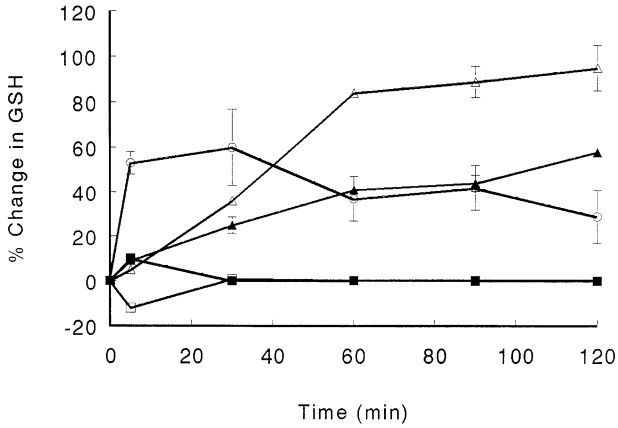


FIG. 3. Percentage increase in the intracellular GSH concentration in rat lung slices (25–30 mg) incubated in Krebs physiological salt solution (5.0 mL, pH 7.40) containing 1.0 mM of either: GSH (○), GSH isopropyl diester (△), γ-glu-cys isopropyl diester (▲), GSH isopropyl monoester (□) and γ-glu-cys isopropyl monoester (■). Each point represents triplicate incubations with three animals mean ± SEM.

same retention times. Two solvents were run simultaneously, solvent A, which was 0.25% HPLC glacial acetic acid in HPLC grade water (Rathburn Chemicals Ltd, Walkerburn, Scotland) adjusted to pH 3.70, and solvent B, which was HPLC grade acetonitrile. The flow rate was 1.5 mL min^{-1}. A 10 μL sample was injected and run on a mobile phase of 92% A and 8% B for 4.5 min changed to 15% A and 85% B over 1 min. This continued for 7 min, at which point the gradient returned to 92% A and 8% B over 1.5 min. Both changes of the solvent mixture in the mobile phase followed a linear gradient program. Five–min equilibration time was given before the next injection.

RESULTS

The uptake of the thiols were determined in rat lung slices and trachea sections by using HPLC to measure thiol levels. The effect of the carboxylesterase inhibitor, BNPP, upon L-CIPE and D-CIPE uptake and metabolism was determined. Triplicate incubations were carried out at each time point for each animal and three animals were used for each compound. Results are expressed as the concentration of thiol per mg wet wt of tissue.

UPTAKE AND METABOLISM OF GSH ESTERS

Intracellular Thiol Concentrations

GSH. Because the intracellular concentration of GSH varied from the lungs of different animals, the data have been normalised and expressed as a percentage change in the GSH concentration compared to the control at T = 0 (Fig. 3, Table 1). In control slices, GSH concentrations averaged 1.55 ± 0.21 (±SEM) nmol mg^{-1} and remained elevated for at least 60 min and, in most cases, for 120 min. However, depending upon the integrity of the slices, the concentration did fall by up to 40% in a limited numbers of slices. Slices incubated with GSH isopropyl diester had intracellular GSH concentration increased by 84% at 60 min, which continued to increase slowly up to 95% at 120 min. Incubation of slices with γ-glu-cys isopropyl diester produced a steady increase in the GSH concentration so that at 120 min, the concentration had increased by 58%. GSH isopropyl monoester and γ-glu-cys isopropyl monoester did not increase the intracellular concentration of GSH above control slice values. Slices incubated with GSH showed a rapid increase in GSH concentrations at 5 and 30 min (53 and 60%, respectively) which then fell, but remained above control levels from 60–120 min (e.g. between 29–42%).

CySH. In control slices, the concentration of L-CySH averaged 0.25 ± 0.03 (±SEM) nmol mg^{-1}, which remained steady for at least 60 min and then fell with time to undetectable levels by 120 min. Slices incubated with γ-glu-cys isopropyl diester showed a steady increase in the intracellular CySH concentration which then remained steady from 60 min onward (0.85–0.91 nmol mg^{-1}, Fig. 4, Table 1). Incubation of slices with GSH isopropyl monoester and GSH isopropyl diester raised CySH concentrations to a maximum at 30 and 60 min, respectively, which then decreased with time (max values 0.56 ± 0.04 and 0.65 ± 0.11 nmol mg^{-1}, respectively). GSH and γ-glu-cys isopropyl monoester did not increase the intracellular concentration of CySH.

ESTERS. In slices incubated with GSH isopropyl diester and γ-glu-cys isopropyl diester, the concentration of the unhydrolysed esters reached a steady state within 5 min (1.17–1.28 nmol mg^{-1} and 0.35–0.45 nmol mg^{-1}, respectively, Fig. 5, Table 1). GSH isopropyl monoester reached a max at 30 min (1.37 ± 0.15 nmol mg^{-1}) and then decreased with time. γ-glu-cys isopropyl monoester was not detected intracellularly.

γ-GLU-CYS. In slices incubated with γ-glu-cys isopropyl diester, the concentration of γ-glu-cys increased from undetectable levels at 0 and 5 min up to a maximum of 1.29 ± 0.05 nmol mg^{-1} wet wt at 120 min (Table 1). The other esters, including γ-glu-cys isopropyl monoester, did not increase the γ-glu-cys concentration, which remained undetectable throughout the incubation.

Extracellular Thiol Concentrations

The rates of disappearance of GSH and the four esters from the extracellular medium with the slowest first was in the order of: GSH > GSH isopropyl diester > γ-glu-cys isopropyl diester > γ-glu-cys isopropyl monoester > GSH isopropyl monoester (Fig. 6, Table 2). The extracellular concentration of GSH had decreased by 15% at 120 min, GSH isopropyl diester had decreased by 27%, γ-glu-cys isopropyl diester by 66% and both monoesters by over 90% at 120 min.

TABLE 1. Intracellular lung concentrations of CySH, GSH, esters and γ-glu-cys following incubation of lung slices with either GSH, GSH isopropyl monoester, GSH isopropyl diester, γ-glu-cys isopropyl monoester and γ-glu-cys isopropyl diester

Cysteine	Time (min)	GSH	GSH isopropyl diester	γ-glu-cys isopropyl diester	GSH isopropyl monoester	γ-glu-cys isopropyl monoester
	0	0.27 ± 0.03	0.27 ± 0.07	0.25 ± 0.00	0.21 ± 0.01	0.22 ± 0.00
	5	0.28 ± 0.01	0.34 ± 0.05	0.28 ± 0.04	0.39 ± 0.04	0.20 ± 0.01
	30	0.28 ± 0.01	0.47 ± 0.08	0.53 ± 0.05	0.56 ± 0.04	0.20 ± 0.00
	60	0.27 ± 0.01	0.65 ± 0.11	0.85 ± 0.12	0.49 ± 0.07	0.19 ± 0.01
	90	0.23 ± 0.01	0.50 ± 0.11	0.86 ± 0.13	0.29 ± 0.04	0.13 ± 0.01
	120	0.18 ± 0.01	0.43 ± 0.11	0.91 ± 0.11	0.24 ± 0.05	0
Glutathione						
	0	1.83 ± 0.22	2.00 ± 0.10	1.40 ± 0.40	1.18 ± 0.14	1.21 ± 0.04
	5	2.79 ± 0.24	2.09 ± 0.14	1.53 ± 0.20	1.04 ± 0.16	1.33 ± 0.03
	30	2.93 ± 0.28	2.72 ± 0.17	1.75 ± 0.26	1.20 ± 0.15	1.20 ± 0.05
	60	2.50 ± 0.26	3.67 ± 0.12	1.98 ± 0.14	1.19 ± 0.12	1.22 ± 0.08
	90	2.60 ± 0.24	3.77 ± 0.29	2.02 ± 0.17	1.18 ± 0.15	1.23 ± 0.10
	120	2.36 ± 0.28	3.89 ± 0.41	2.21 ± 0.08	1.10 ± 0.07	1.19 ± 0.07
Ester						
	0	—	0	0	0	0
	5	—	1.18 ± 0.06	0	0.58 ± 0.30	0
	30	—	1.28 ± 0.04	0.37 ± 0.16	1.37 ± 0.15	0
	60	—	1.31 ± 0.06	0.39 ± 0.18	1.07 ± 0.10	0
	90	—	1.17 ± 0.07	0.35 ± 0.13	0.33 ± 0.09	0
	120	—	1.27 ± 0.07	0.45 ± 0.23	0.23 ± 0.06	0
γ-glutamylcysteine						
	0	0	0	0	0	0
	5	0	0	0	0	0
	30	0	0	0.55 ± 0.08	0	0
	60	0	0	0.90 ± 0.09	0	0
	90	0	0	1.00 ± 0.02	0	0
	120	0	0	1.29 ± 0.05	0	0

All values are expressed in nmol mg^{-1} tissue, wet wt and are presented as mean ± SEM, $N = 3$.

UPTAKE AND METABOLISM OF L- AND D-CySH

Trachea

Incubation of tracheal sections with either L- or D-CySH (1.0 mM) at 37° increased the intracellular concentrations of both monoesters, to reach a steady state from 15 min onward with concentrations ranging between 1.35–1.56 nmol mg^{-1} and 0.96–1.23 nmol mg^{-1}, respectively (Fig. 7A). Reducing the incubation temperature to 4° had little effect on the accumulation of D-CySH but L-CySH levels

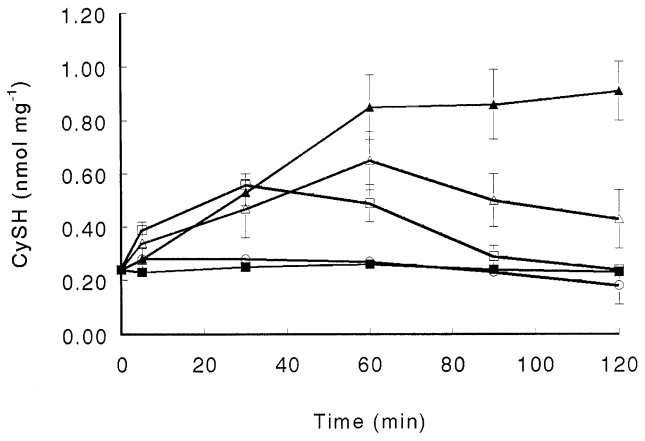


FIG. 4. Increase in the intracellular CySH concentrations in rat lung slices (25–30 mg) incubated in Krebs physiological salt solution (5.0 mL, pH 7.40) containing 1.0 mM of either: GSH (○), GSH isopropyl diester (△), γ-glu-cys isopropyl diester (▲), GSH mono-isopropyl ester (□) or γ-glu-cys isopropyl monoester (■). Each point represents triplicate incubations with three animals mean ± SEM.

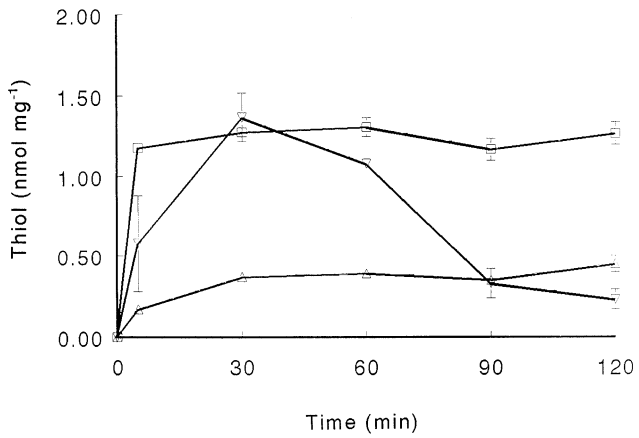


FIG. 5. Increase in the intracellular GSH ester concentrations in rat lung slices (25–30 mg) incubated in Krebs physiological salt solution (5.0 mL, pH 7.40) containing 1.0 mM of the selected GSH ester. GSH isopropyl diester (□), γ-glu-cys isopropyl diester (△), GSH isopropyl monoester (▽). Each point represents triplicate incubations with three animals mean ± SEM.

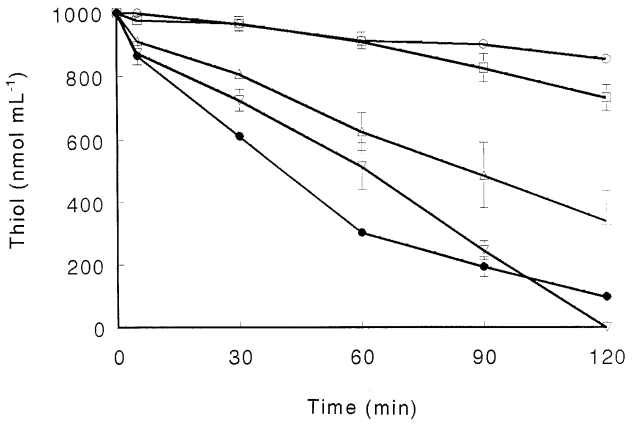


FIG. 6. Disappearance of GSH and GSH esters (1.0 mM) from the extracellular medium containing rat lung slices (25–30 mg) in Krebs physiological salt solution (5.0 mL, pH 7.40). GSH (○), GSH isopropyl diester (□), γ-glu-cys isopropyl diester (△), GSH isopropyl monoester (▽) and γ-glu-cys isopropyl monoester (●). Each point represents triplicate incubations with three animals mean ± SEM.

continued to increase up to 90 min (0.96 ± 0.11 nmol mg^{-1} at 37° and 2.73 ± 0.18 nmol mg^{-1} at 4°, Fig. 7A).

Lung

Lung slices incubated with L- or D-CySH (1.0 mM) at 37° increased the intracellular concentrations of both monoesters to a steady state from 15 min onward. D-CySH levels were approximately two fold higher than L-CySH levels throughout the incubation and levels ranged between 1.83–2.25 and 0.88–1.25 nmol mg^{-1} respectively (Fig. 7B). Reducing the temperature to 4° had little effect on the accumulation of D-CySH, but L-CySH levels increased progressively throughout the experiment such that 90 min the levels were 2.63 ± 0.13 nmol mg^{-1} at 4° compared with 1.06 ± 0.1 nmol mg^{-1} at 37° (Fig. 7B).

UPTAKE AND METABOLISM OF L- AND D-CIPE

Trachea

Incubation of tracheal sections with L- or D-CIPE (1.0 mM) increased the intracellular concentrations of L- and D-

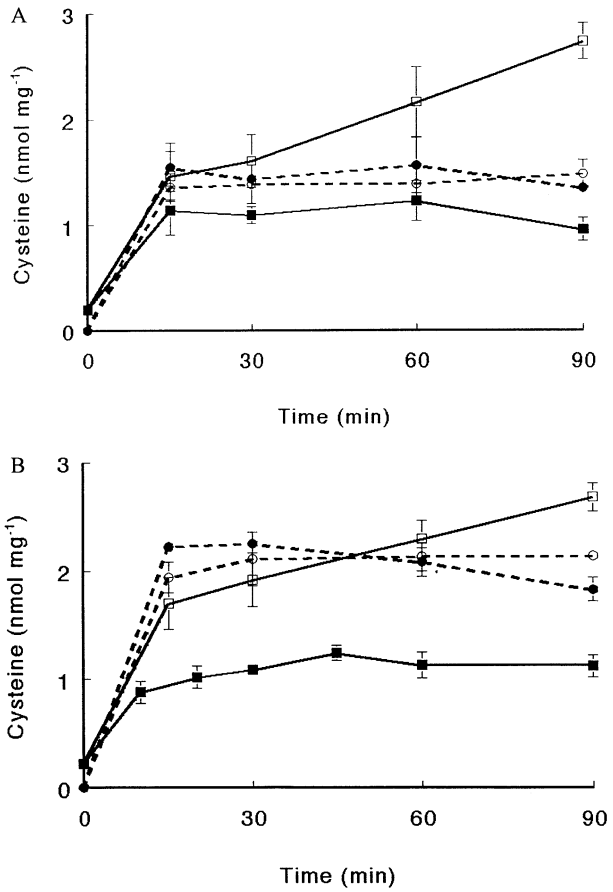


FIG. 7. Intracellular elevation of D- and L-CySH in A) rat trachea sections (15–20 mg) and B) rat lung slices (25–30 mg) following incubation in Krebs physiological salt solution (pH 7.40) containing the respective isomer (1 mM) at 4° and 37°. Each point represents triplicate incubations with three animals mean ± SEM. L-CySH, 37° (■); L-CySH, 4° (□); D-CySH, 37° (●); D-CySH, 4° (○).

CySH, respectively. L-CySH levels increased up to 30 min and remained steady for the duration of the incubation (5.16–5.56 nmol mg^{-1}), whereas D-CySH concentrations increased to reach 11.09 nmol mg^{-1} at 90 min (Fig. 9A). Extracellularly, the rate of disappearance of D-CIPE was approximately half that of L-CIPE, e.g. at 90 min D-CIPE levels had decreased by 32.7% and L-CIPE by 66.7%.

TABLE 2. Extracellular concentrations of GSH, GSH isopropyl monoester, GSH isopropyl diester, γ-glu-cys isopropyl monoester and γ-glu-cys isopropyl diester from the medium containing lung slices

Time (min)	GSH	GSH isopropyl diester	γ-glu-cys isopropyl diester	GSH isopropyl monoester	γ-glu-cys isopropyl monoester
0	1008 ± 10	1006 ± 8	994 ± 12	1003 ± 11	1000 ± 6
5	999 ± 3	976 ± 25	909 ± 12	872 ± 36	864 ± 12
30	964 ± 25	965 ± 22	807 ± 17	724 ± 34	612 ± 16
60	910 ± 26	907 ± 5	625 ± 60	516 ± 74	303 ± 19
90	898 ± 14	824 ± 44	487 ± 105	249 ± 29	193 ± 32
120	851 ± 13	731 ± 40	341 ± 97	0	97 ± 17

All values are expressed in nmol mg^{-1} tissue, wet wt and are presented as mean ± SEM, $N = 3$.

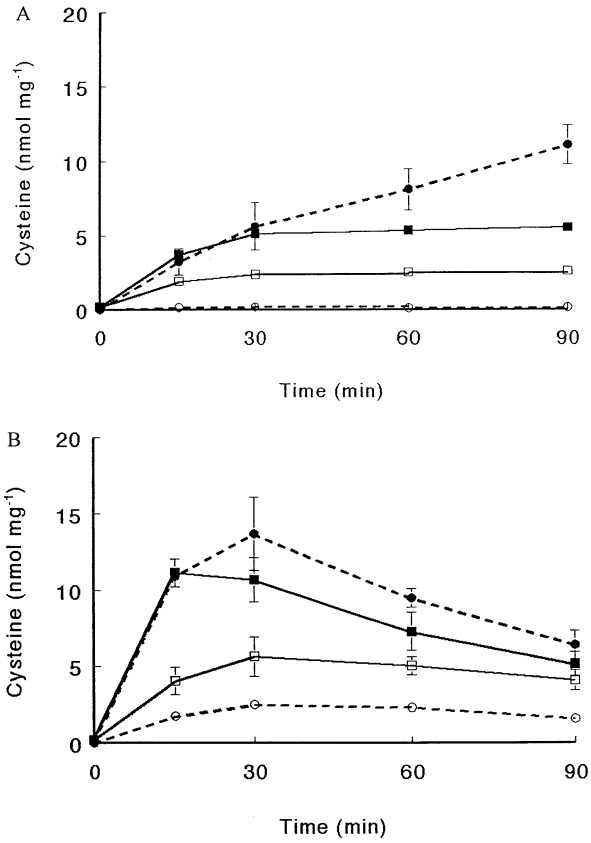


FIG. 8. Disappearance of D- and L-CIPE from A) the trachea section medium containing trachea sections and trachea sections preincubated with BNPP and B) disappearance of D- and L-CIPE from the lung slice medium containing normal lung slices and lung slices preincubated with BNPP. Each point represents triplicate incubations with three animals mean ± SEM. L-CIPE (■); L-CIPE [BNPP] (□); D-CIPE (●); D-CIPE [BNPP] (○).

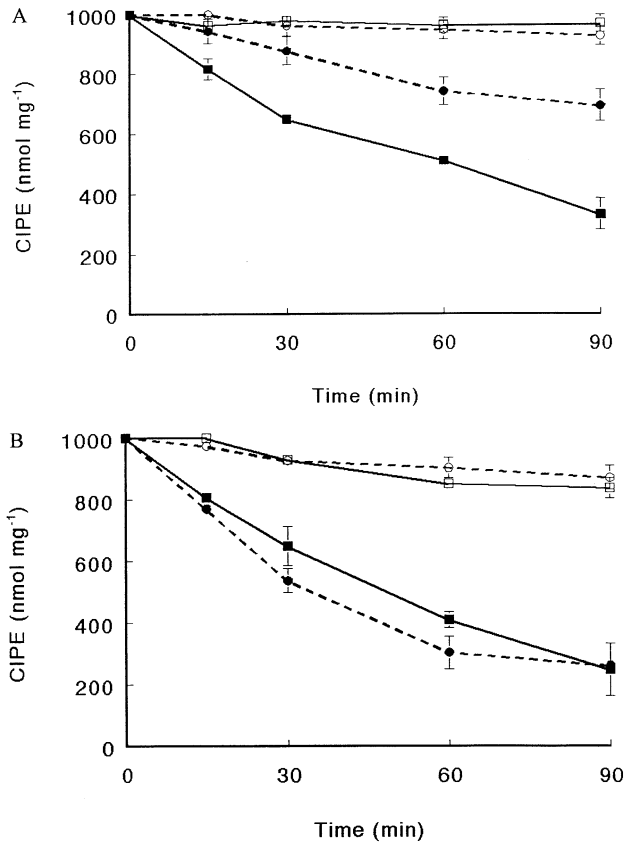


FIG. 9. Intracellular elevation of D- and L-CySH in A) rat tracheal sections (15–20 mg) and B) rat lung slices (25–30 mg) preincubated with Krebs physiological salt solution (pH 7.40) or BNPP for 30 min prior to incubation with D- or L-CIPE (1 mM). Each point represents triplicate incubations with three animals mean ± SEM. L-CySH (■); L-CySH [BNPP] (□); D-CySH (●); D-CySH [BNPP] (○).

The Effect of BNPP

Preincubation with the carboxylesterase inhibitor BNPP almost completely eliminated the uptake of D-CySH into the tracheal sections (0.12–0.17 nmol mg^{-1}) and reduced L-CySH concentrations by approximately two-fold (1.92–2.40 nmol mg^{-1}, Fig. 8A). Extracellularly, the rate of disappearance of the esters was reduced dramatically: L-CIPE levels had decreased by only 2.5% at 30 min and D-CIPE by 4.0% at 30 min and by 8.0% at 90 min (Fig. 9A).

Lung

Incubation of lung slices with L- or D-CIPE (1.0 mM) increased the intracellular concentrations of L- and D-CySH, respectively. Intracellular levels of L- and D-CySH followed similar profiles, reaching a peak and then falling (11.1 nmol mg^{-1} at 15 min and max 13.7 nmol mg^{-1} at 30 min, Fig. 8B). The extracellular rates of disappearance of L- and D-CIPE from the incubation medium were very similar and decreased 75.2% and 74.0%, respectively, at 90 min (Fig. 9B).

The Effect of BNPP

BNPP reduced the concentration of intraslice L- and D-CySH and altered their uptake profiles. However, the intraslice concentrations of D-CySH were reduced to a much greater degree than were the L-CySH concentrations after BNPP treatment. The intracellular concentrations of L- and D-CySH peaked at 30 min with levels of 5.6 and 2.4 nmol mg^{-1} and then remained steady (Fig. 8B). The rate of disappearance of L- and D-CIPE from the incubation medium was reduced by a similar amount: the concentrations had decreased by 16.2% and 12.8% at 90 min, respectively (Fig. 9B).

DISCUSSION

The cellular GSH levels in $P388D_1$ macrophages and bovine pulmonary artery endothelial cells were elevated following incubation with GSH [16, 17]. In this study, we have shown that rat lung slices incubated with GSH also had increased cellular GSH concentrations, which maybe due to the extracellular catabolism of GSH by γ-glutamyl transpeptidase, followed by uptake and resynthesis of the amino acids. However, the increase in the cellular GSH concentration is rapid and there are two other possible

routes by which intact GSH might enter the lung slices at a faster rate. The first is a Na^+ dependent transport system, which is known to operate in rat alveolar epithelial cells, and the second is a bidirectional GSH transporter, which at the concentrations of GSH used here would favour uptake of GSH [2–4]. It is possible that some of the rapid uptake of GSH in the rat lung slices may be attributed to direct uptake of GSH by either or both of these systems.

Significant protection from oxidative injury occurred in cells that were able to transport intact GSH from an exogenously supplied pool of GSH in the medium [2–4]. However, GSH does not readily cross the plasma membrane intact in most cells because all the major solution species are ionised. Esterification of the molecule has been used as a way of raising intracellular concentrations of GSH, and it was suggested that the effectiveness of GSH ethyl monoester was the absence of a negatively charged group on the glycine residue and an increased lipophilicity [16–24]. However, ethyl monoesters still have ionised species, whereas ethyl diesters are not ionized at all, which may lead to more efficient transport across the cell membrane. GSH ethyl diester was more effective at delivering GSH into human cells and mouse $P388D_1$ cells than either GSH or GSH ethyl monoester and was a more potent antidote to 1-chloro-2,4-dinitrobenzene and menadione toxicity in mouse $P388D_1$ cells [17–19]. GSH ethyl diester is believed to cross the plasma membrane by diffusion and undergo hydrolysis to yield GSH ethyl monoester intracellularly, which is then hydrolysed at a slower rate to yield GSH, thus bypassing the feedback inhibition of γ-glu-cys synthetase [24]. GSH esters are also more resistant to hydrolysis by γ-glutamyl transferase [17–19].

Following administration of either GSH ethyl mono- or diester to mice, GSH levels in the kidney and liver were similar. However, in the hamster, liver GSH concentrations were higher following administration of GSH ethyl diester in contrast to GSH ethyl monoester. The difference in the metabolism would appear to be due to the presence of a plasma esterase in the mouse (also found in the rat) that can hydrolyse GSH ethyl diester to GSH ethyl monoester, which is absent from the hamster (as well as from the guinea pig and human) [24]. Plasma esterase activities are 20–100 times higher in the mouse than in the human; however, in esterase deficient mice the half–lives of GSH derivative diesters are very similar [25]. Species such as esterase deficient mice or species that lack the plasma esterase may prove useful in relating the pharmacokinetic and pharmacodynamic studies of GSH diesters to the human.

In our studies, by using rat lung slices incubated with GSH isopropyl diester or γ-glu-cys isopropyl diester had increased cellular concentrations of GSH, CySH and unhydrolysed isopropyl diester. However, neither of the respective isopropyl monoesters were detected which would indicate that rat lung does not possess an esterase capable of hydrolysing isopropyl diesters to isopropyl monoesters, but instead hydrolyses isopropyl diesters directly to GSH. Preliminary results using an homogenate of rat lung suggest that hydrolysis of GSH isopropyl diester is carried out by enzymes insensitive to BNPP, unlike CySH esters which are hydrolysed primarily by BNPP sensitive serine esterases.

The rate of disappearance of isopropyl diesters from the external medium was much slower than the disappearance of isopropyl monoesters, indicating that they are more resistant to hydrolysis, making them more suited to deliver cellular nucleophiles over a longer time period than the CySH esters used previously [7, 13]. The rapid disappearance of the isopropyl monoesters from the external medium was not matched with an increase in cellular GSH and it is possible that hydrolysis of the monoesters to GSH is not the main route of metabolism. Cysteine can autoxidise in the presence of a suitable catalyst, such as a redox metal ion, and it has been shown that copper ions promoted the oxidation of GSH ethyl monoester but not GSH ethyl diester [24, 26]. It is possible that the addition of metal ion chelators may help prolong the half–lives of the monoesters in the medium; however, spontaneous disappearance of any of the isopropyl mono- or diesters was not observed in Krebs alone and it would appear that the presence of the lung slices is needed for the disappearance.

L-CySH is transported almost entirely as a neutral amino acid by one of three neutral amino acid transport systems, designated A, ASC and L. A and ASC are Na^+ dependent and L is Na^+ independent, all of which operate in the direction of the concentration gradient [27]. In rat kidney cortex slices and rat lung slices, L-CySH uptake is dependent upon temperature and is mediated by a single Na^+–dependent uptake process resembling the ASC system. By lowering the temperature, the intracellular concentration of L-CySH is increased, probably because the efflux of L-CySH is slowed by a greater amount than the influx [28, 29].

In this study, we have shown that rat lung slices and tracheal sections incubated with L-CySH had increased cellular concentrations of L-CySH at a reduced temperature of 4°. Reducing the temperature of rat lung slices and tracheal sections incubated with D-CySH had little effect on the uptake of D-CySH. This would indicate that the transport of L-CySH, but not that of D-CySH, is mediated by a temperature–sensitive system resembling the ASC system in rat lung and trachea.

Lung slices incubated with D- and L-CIPE had similar rates of ester disappearance and similar intracellular concentrations of D- and L-CySH. The carboxylesterase inhibitor, BNPP, reduced the intraslice concentration of D- and L-CySH and the rate of disappearance of D- and L-CIPE from the medium, indicating that the enzyme(s) responsible for the hydrolysis of the CySH esters are mediated by carboxylesterases, which do not appear to be stereospecific in the lung.

The situation in the trachea appears different when the rate of disappearance of D-CIPE from the medium is approximately 2-fold slower than L-CIPE. BNPP inhibited the uptake and the rates of disappearance of both D- and

L-CIPE and, in particular, the intracellular concentration of D-CySH was greatly reduced by BNPP. This would indicate that in the trachea, unlike the lung, the enzyme(s) responsible for the hydrolysis of the esters are stereospecific carboxylesterases that have a higher affinity for the L-form.

The carboxylesterase inhibitor, BNPP, acts by irreversibly phosphorylating the active sites of carboxylesterases [12]. In the lung and trachea, BNPP significantly reduced the uptake of D- and L-CySH and the rates of disappearance of D- and L-CIPE, indicting that the main enzyme(s) involved in the metabolism of the esters are type "B" serine hydrolases in the scheme proposed by Aldridge [30]. Carboxylesterase activity is very high in the liver, but the enzymes are widespread in many tissues including the lung and we have demonstrated carboxylesterase activity not only in rat lung, but also in the rat trachea [31].

Previously, we had reported that incubation of lung slices with CySH esters led to greater concentrations of CySH inside the slice than incubation with CySH itself [13]. Extracellular hydrolysis of the ester and subsequent uptake of CySH into the slice is not the major uptake mechanism and although our results are consistent with the hypothesis that CySH esters diffuse into the lung slices and are rapidly hydrolysed intracellularly, producing a concentration gradient down which further diffusion can take place, one cannot exclude the involvement of a membrane bound transport system.

In this study, we have demonstrated that GSH isopropyl diester and γ-glu-cys isopropyl diester elevated GSH concentrations in rat lung slices to a level above that of the feedback inhibited level. GSH itself also proved to be effective at enhancing intracellular concentrations, but GSH isopropyl monoester and γ-glu-cys isopropyl monoester were without effect. Rat lung and trachea have a similar temperature sensitive uptake mechanism for L-CySH but not for D-CySH. Carboxylesterases appear to have a major influence on the uptake and metabolism of D- and L-CIPE by rat lung slices and tracheal sections, but this mechanism appears to be stereospecific in the trachea and not the lung.

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