Pulmonary Toxicity of Thioureas in the Rat

by Angus M. Scott,* Gillian M. Powell,* David G. Upshall,† and C. Gerald Curtis*

Administration of α-naphthylthiourea (ANTU) to rats causes damage to pulmonary endothelial cells and possibly mesothelial lining cells that together may account for the massive pleural effusion characteristic of thiourea toxicity. Using ^{35}S-thiourea as a model compound, the extent of binding of ^{35}S to lung proteins correlated well with the extent of edema, suggesting that the extent of binding of thiourea metabolites is a measure of lung toxicity. ANTU and phenylthiourea (PTU) compete for ^{35}S binding to lung slices, suggesting that these toxins may act in a similar way. Binding of ^{35}S in lung slices from resistant rats is much less than in controls, and resistance cannot be explained by differences in either whole body metabolism or redistribution of thiourea *in vivo*. Lung glutathione levels (*in vitro* and *in vivo*) in normal and resistant rats following thiourea administration were essentially the same. However, at doses of thiourea that cause pleural effusion, there was an increase in total lung glutathione.

Introduction

The thioureas cause massive pulmonary edema and pleural effusion in rats (1–4). One of them, α-napthyl-thiourea (ANTU) was developed as a rat poison, but during extensive trials (4) it became apparent that rats ingesting small nonlethal doses of ANTU rapidly became resistant to it (tachyphylaxis). This resistance reduced the effectiveness of such compounds as potential rodenticides but nevertheless provided a useful model to investigate their mode(s) of action.

Ultrastructural studies have shown high specificity of thioureas for pulmonary endothelial cells (5,6), and even at high doses (50 mg/kg) there appears to be little damage to any other cells in the alveolar capillary unit, although the edema may be extensive (6). In addition, ANTU-induced biochemical changes in levels of angiotensin converting enzyme (7–9) and 5-hydroxytryptamine uptake (10) are considered to reflect specific endothelial cell damage.

Massive pleural effusion is a characteristic feature of thiourea toxicity. It is possible that the intrathoracic pressure exerted on the lung by this effusate is often the cause of death, rather than the alveolar edema that also occurs. Despite extensive research, the mode of action of these compounds remains unclear, although there is good evidence to suggest that the thioureas require metabolic activation before toxic effects are manifested (11–16). Although the mechanism of activation has not been resolved, thiols protect against thiourea toxicity and covalent binding (17), and glutathione depletion enhances both the toxicity and protein binding of ANTU (11) and thiourea (15). Further, administration of either glutathione or cysteine protects against the toxicity of phenylthiourea (PTU) *in vivo* (18). It has also been shown that glutathione can protect against the binding of radioactivity in studies with ^{14}C-thiourea *in vitro* (15). Binding studies with ^{14}C-thiourea (13–17) have demonstrated the covalent binding of radiolabel to proteins in lung and liver, and that binding is less in resistant rats. It has also been shown that radiolabeled ANTU binds covalently to protein in the lung and liver (11,12). Interestingly, between two and six times the binding is observed if ANTU is labeled with ^{35}S rather than ^{14}C (11,12), which suggests that binding of ^{35}S is a better measure of total binding. No binding studies, however, have been undertaken with ^{35}S-thiourea.

In this study, three aspects of thiourea toxicity are explored: namely *a*) the effect of ANTU on the ultrastructure of the rat lung with particular attention focused on the mesothelial layer that lines the surface of the lung, *b*) the binding of ^{35}S (from ^{35}S-thiourea) to tissue proteins *in vivo* and *in vitro* in normal and thiourea-resistant rats to determine if there is a correla-

*Department of Biochemistry, University College Cardiff, P.O. Box 78, Cardiff CF1 1XL, UK.

†Chemical Defence Establishment, Porton Down, Salisbury, Wilts, SP40 0JQ, UK.

Address reprint requests to C. G. Curtis, Department of Biochemistry, University College Cardiff, P.O. Box 78, Cardiff CF1 1XL, UK.

tion between toxicity and binding, and c) the levels of glutathione in lungs of normal and resistant rats.

Materials and Methods

Chemicals

All chemicals used were Analar grade unless stated otherwise. α-Naphthylthiourea was a gift from the Chemical Defence Establishment (Porton Down, Salisbury, UK).

Radiochemicals

[35]S-Thiourea (25 or 60 mCi/mmole) was obtained from Amersham International (Bucks, UK).

Experimental Animals

MRC hooded rats (300–400 g) were used throughout the study and were fed on diet 41B (Pilsbury Ltd., Edgbaston, Birmingham, UK).

Development of Thiourea Resistance

Animals received 0.5 mg of thiourea/kg body weight in saline (0.15 M, 0.3 mL) IP daily for 3 days. Control rats were injected with saline (0.15 M) alone. Treated rats were designated resistant; control were designated normal. Experiments were carried out 24 to 48 hr after the final injection.

Electron Microscopy

Rats, under ether anesthesia, received pentobarbitone (60 mg/kg) IV via the penal vein. The trachea was isolated and cannulated (Portex, Hythe, Kent, UK; int. dia. 1.67 mm; ext. dia. 2.42 mm). After a midline abdominal incision, a small incision was made in the diaphragm to deflate the lungs. The thoracic cavity was exposed and the lungs were fixed by the method of Hammond and Mobbs (19). Formalin vapor was instilled via the trachea cannula until the lungs were fully inflated. The lungs were removed, the trachea was clamped, and the whole preparation was immersed in phosphate-buffered formalin (pH 7.4) for 72 hr. Blocks of tissue (approximately 1 mm³) were cut from two areas of the lung—from the edge containing the mesothelial layer and from central areas of lung parenchymal tissue. These blocks were stained with uranyl acetate (2%) for 60 min in the dark at 4°C, and dehydrated in ethanol followed by propylene oxide, and embedded in araldite. Sections (60–90 nm) were cut using a Reichart OM4 microtome and mounted on a celloidine-carbon coated grid. Sections were stained in 5% uranyl acetate followed by Reynold's lead citrate. Sections were examined in a Philips EM 400T electron microscope at 80 KV acceleration voltage.

Preparation of Lung Slices

Animals were anesthetized as described above and exsanguinated. The lungs were deflated and the thoracic cavity was exposed. An incision was made in the right ventricle of the heart and a cannula (Portex; int. dia. 1.67 mm; ext. dia. 2.42 mm) was inserted into the pulmonary artery. Isotonic saline was perfused into the lung at a pressure of 25 cm H_2O, and an incision made in left auricle allowed the exit of saline from the lungs. This procedure cleared the vascular bed. Following perfusion, the lungs were dissected free from the heart, esophagus, and associated connective tissue. The external surfaces of the lungs were rinsed in ice-cold saline (0.15 M NaCl). Slices (0.7 mm) were prepared using the McIllwain tissue chopper and incubated in MEM Eagles medium (200 mg tissue/mL medium) in a shaking water bath (37 °C) with continuous gassing (95% O_2, 5% CO_2). Slices were equilibriated for 30 min and then suspended in fresh medium.

Preparation of Tissues for Binding Studies (In Vivo)

Animals were anesthetized with ether, and pentobarbitone (60 mg/kg) was administered IV via the penal vein. Following a midline abdominal incision, the dorsal aorta was isolated and clamped distal to the renal artery. A cannula (int. dia. 1.67 mm; ext. dia. 2.42 mm) was inserted into the dorsal aorta and saline (0.15 M NaCl) was infused via the cannula at a pressure of 1 M H_2O. An incision was made in the vena cava immediately anterior to the diaphragm to allow the saline to escape. This procedure cleared the liver and kidneys of blood. The lungs were also perfused free of blood as described previously. Lungs, liver, and kidneys were removed, weighed, and assayed for protein-bound radioactivity.

Assay for Protein-Bound [35]S

Tissue homogenates (20%) were prepared in ice-cold saline (0.15 M NaCl). Five volumes of ice-cold trichloracetic acid (TCA) (20% w/v) containing phosphotungstic acid (2% w/v) were added to one volume of tissue homogenate, the samples were kept on ice for 30 min and centrifuged (600g, 5 min). The supernatant was discarded and the pellet resuspended in five volumes of TCA (20%). This step was repeated three times or until radioactivity was not detectable in the supernatants. The washed pellet was then dissolved in 2.5 M NaOH at 60°C for 4 hr. Protein in the solubilized pellet was determined by the method of Lowry et al. (20). Radioactivity was determined by scintillation counting (LKB Rackbeta 1217) with quench curve correction by the external standard ratio method.

Measurement of Glutathione

Tissue homogenates (3% w/v) were prepared in ice-cold EDTA (30 μM). Aliquots (1 mL) were filtered using Centricon 10K filters (Amicon) by centrifuging (600g for 30 min at 4°C). The filtrates were diluted 10 times with EDTA (30 μM) and assayed for glutathione by the o-phthalaldehyde fluorometric assay (21) using a Hitachi-Perkin Elmer MPF-2A fluorescence spectrophotometer.

Actual amounts of glutathione were determined using a standard curve.

Experimental and Results

Effect of ANTU on Lung Ultrastructure

Three rats received a single IP injection of ANTU (20 mg/kg body weight) dispersed in olive oil (0.3 mL), and three rats (designated control) received olive oil alone. Electron micrographs of the alveolar capillary unit from controls (Fig. 1A) showed no edema in the alveolar spaces. Moreover, Type I epithelial cells, capillary endothelial cells, and the basement membrane all appeared intact. By contrast, the micrograph obtained from ANTU-treated lungs is shown in Figure 1B. The alveolar space is flooded with fluid and protein, and the endothelial barrier appears absent, whereas the basement membrane and Type I cells appear intact and unaffected. In addition, changes were observed in the mesothelial cell layer that lines the outside of the lung. A normal mesothelial cell layer from a control animal in which two mesothelial cells lie on a thin basement membrane, overlying interstitial tissue containing collagen bundles is shown in Fig. 2A. After ANTU challenge (Fig. 2B) the junctions between the cells appear intact; the underlying basement membrane, however, is grossly swollen.

Binding of ^{35}S (from Thiourea) to Proteins in Lung, Liver, and Kidneys *In Vivo*

Resistant and normal rats (three of each) were anesthetized with ether and received ^{35}S-thiourea (15 mg/kg body weight, 7 μCi/mmole) in 0.15 M NaCl (0.3 mL) IP. After 4 hr the animals were anesthetized and the vascular beds of liver, lungs, and kidneys were cleared of blood. Each tissue was assayed for protein-bound ^{35}S. In normal rats, protein binding was highest in lung; however, binding was approximately 60% less in resistant rats (Fig. 3). In this study resistance was developed by repeated administration of low doses of nonradioactive thiourea. It was, therefore, possible that unlabeled drug was occupying binding sites in the tissues of the resistant rats. The subsequent binding of ^{35}S thiourea would then show an apparent decrease because some of the binding sites were already occupied. To eliminate this possibility, resistance was developed using ^{35}S-thiourea. Rats were treated with ^{35}S-thiourea (0.5 mg/kg body weight, 5 μCi/mmole) IP daily for 3 days; 24 hr after the final injection the vascular beds of the lungs, liver, and kidneys were cleared of blood and protein-bound ^{35}S was measured. Total binding of ^{35}S under these conditions was relatively low (approximately 0.2% of that observed in normal rats after 4 hr at a dose of 15 mg/kg body weight which causes edema); therefore, the reduced binding in resistant rats could not be explained by the saturation of binding sites with thiourea or its metabolites. In these *in vivo* studies, the

degree of resistance was confirmed by using the ratio of wet weight lung/body weight as an index of edema following thiourea challenge. Resistant rats showed consistently less edema than the normals, and there was a good correlation (correlation coefficient 0.963) between the amount of protein-bound ^{35}S in lung and the degree of edema (Fig. 4).

Binding of ^{35}S (from Thiourea) to Proteins in Lung Slices

Lung slices from two normal rats and two resistant rats were prepared as described. Slices were incubated with ^{35}S-thiourea (0.167 mM, 6 μCi/mmole) for 2 hr. Protein-bound ^{35}S was approximately 65% less in resistant slices than in the normals (Table 1). Equimolar ANTU or PTU inhibited binding in normal slices by 76% and 69%, respectively, whereas urea (the oxygen analogue of thiourea that does not produce edema) had no effect on the binding of ^{35}S. ANTU and PTU also reduced the binding in slices from resistant animals by 44% and 38%, respectively, whereas urea had no effect.

Effect of Thiourea on Lung Glutathione Levels

Rats received IP injections of thiourea (15 mg/kg body weight; in 0.15 M NaCl, 0.3 mL) or 0.15 M NaCl (0.3 mL). At 1 and 2 hr after administration animals were anesthetized, the lungs cleared of blood, and the tissue was assayed for glutathione. Glutathione levels in test and control lungs were the same after 1 hr, but after 2 hr the glutathione levels in thiourea treated rats had risen by 60% (Table 2).

Table 1. The *in vitro* binding of ^{35}S derived from ^{35}S-thiourea to protein in lung slices.[a]

Incubation conditions	Protein binding, pmole ^{35}S-thiourea equivalents/mg protein	
	Normal slices	Resistant slices
Thiourea	217 ± 35	75 ± 5
Thiourea + ANTU	53 ± 3	42 ± 1
Thiourea + PTU	68 ± 15	47 ± 5
Thiourea + urea	243 ± 72	82 ± 9

[a]n = 3. Values are ± SD.

Table 2. Lung glutathione levels following thiourea challenge (15 mg/kg body weight) *in vivo*.[a]

Time, hr	nmole GSH/whole lung		Lung wet weight, g	
	Control	Test	Control	Test
1	311 ± 7	322 ± 46	1.39 ± 0.03	1.59 ± 0.26
2	452 ± 30	764 ± 7	1.42 ± 0.04	3.85 ± 0.65

[a]n = 2; see text for experimental details.
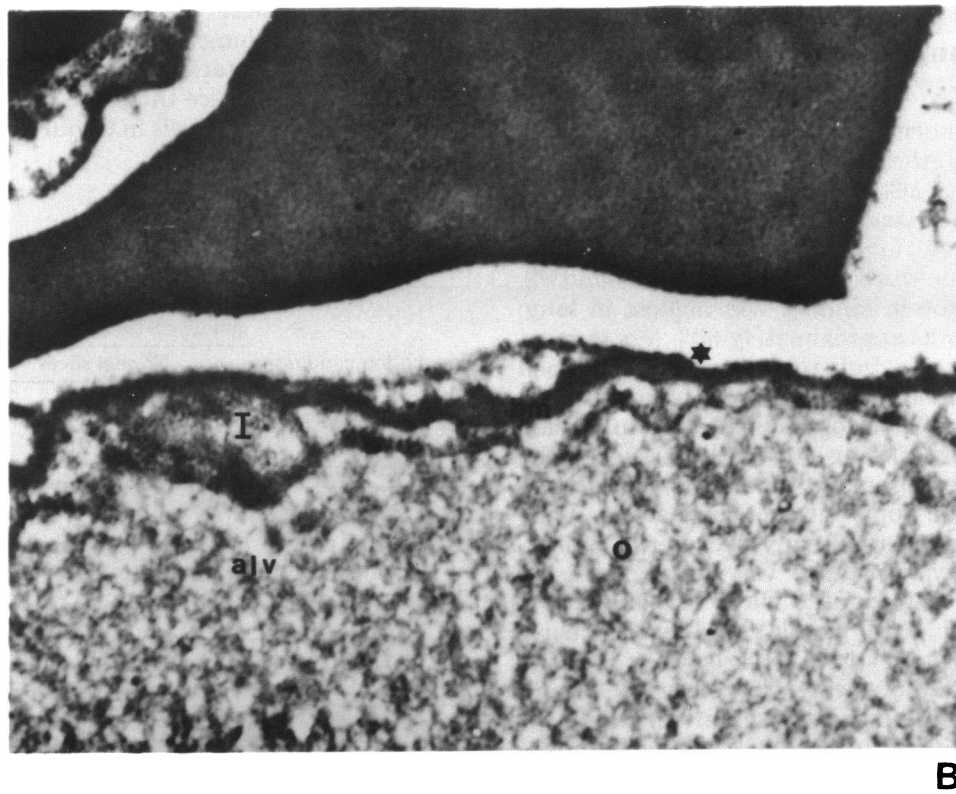
FIGURE 1. (A) Electron micrograph of a typical normal alveolar capillary unit, ×10,000. (B) Electron micrograph of a typical alveolar capillary unit following ANTU challenge (20 mg/kg), ×10,000. Abbreviations: en, endothelial cell; alv, alveolus; I, Type I epithelial cell; o, edematous protein; *, endothelial cell not present.

A

B

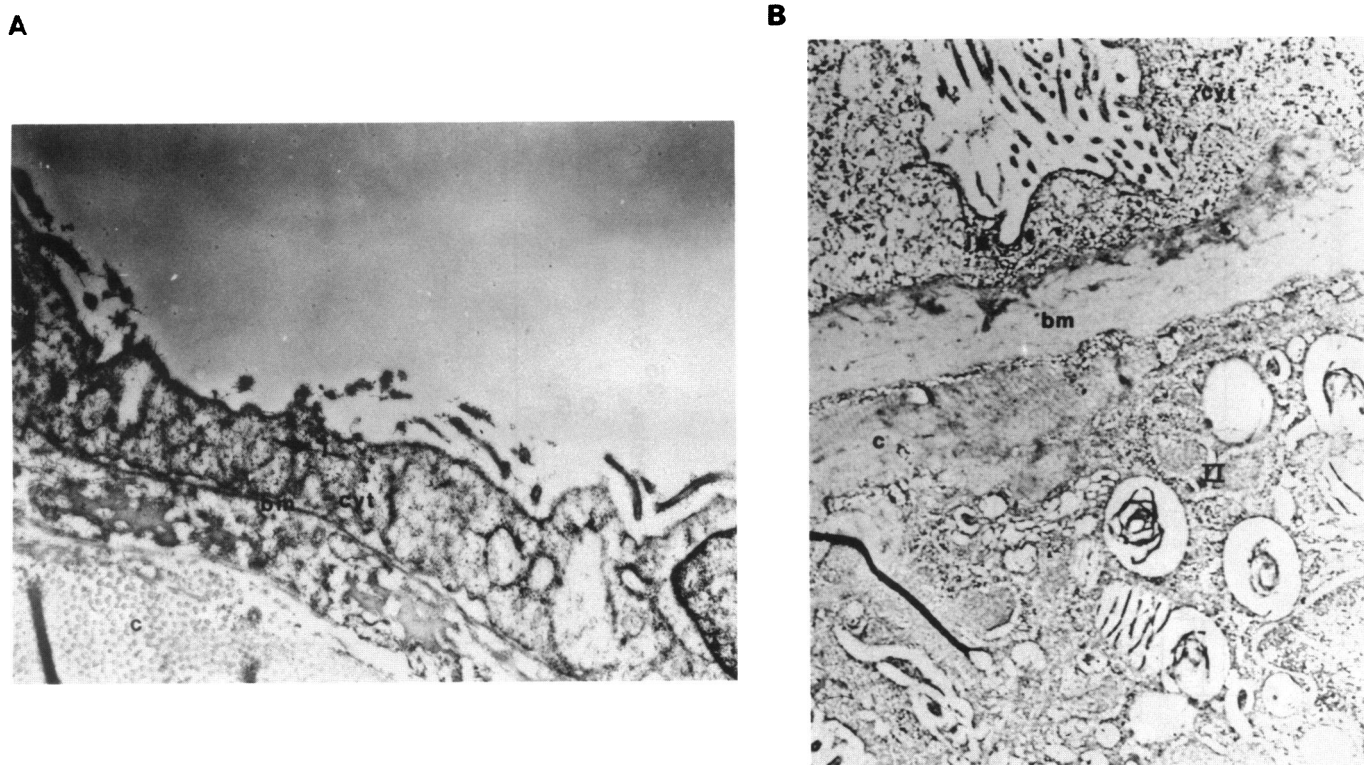FIGURE 2. (A) Electron micrograph of a typical normal mesothelial lining layer, ×10,000. (B) Electron micrograph of a typical mesothelial lining layer following ANTU challenge (20 mg/kg body wt), ×10,000. Abbreviations: cyt, cytoplasm; bm, basement membrane; c, collagen bundles; and II, Type II epithelial cell.

In other experiments, lung slices were prepared from normal and resistant rats and incubated *in vitro* in the presence and absence of 1 mM thiourea. The tissue was sampled at 0, 1, and 2 hr and assayed for glutathione. Glutathione levels were substantially the same in normal and resistant rats at all of the time points (Fig. 5).

In further experiments glutathione levels were measured in lungs from normal and resistant rats 24 hr following the final injection. Levels of glutathione were not altered significantly in resistant rats (normal: 428 ±15 nmole GSH/whole lung; resistant: 460 ±18 nmole GSH/whole lung. Results expressed as an average of two determinations).

Discussion

Many edemagenic agents are reported to cause pulmonary endothelial damage, allowing excessive fluid to invade the interstitium. There are three possible routes for the subsequent escape of the fluid: clearance by lymphatics, passage into the alveolar airways, and passage into the pleural cavity. The majority of edemagenic agents enhance the passage of fluid into alveolar airways, presumably by causing damage to the alveolar epithelial lining, resulting in alveolar edema. However, the thiour-

eas are unusual in causing severe pleural effusion, possibly because of an apparent sensitivity of the mesothelial cell lining layer. This possibility is supported by the results reported here. Evidence is also presented that the extent of binding of the agent or its metabolites is related to edema formation and that thioureas do not cause depletion of glutathione levels. Resistance to the toxins cannot be explained by differential metabolism, tissue distribution, or altered glutathione levels.

SDS-PAGE analysis of the protein composition of the pleural effusion following ANTU induced lung damage has shown that it is qualitatively identical to that of normal plasma (unpublished observations). The passage of this cell-free filtrate into the cavity might be expected to coincide with pulmonary vascular endothelial damage, and this is confirmed by ultrastructural and histochemical examinations in this and other studies with thioureas (5–10). Whether or not endothelial damage alone is sufficient to allow excessive movement of fluid into the pleural cavity is not clear. However, the associated change in the mesothelial basement membrane (grossly swollen) and the lack of any apparent changes to the alveolar epithelial barrier suggests that these particular bloodborne edemagenic agents are atypical in causing pleural effusion as well as alveolar
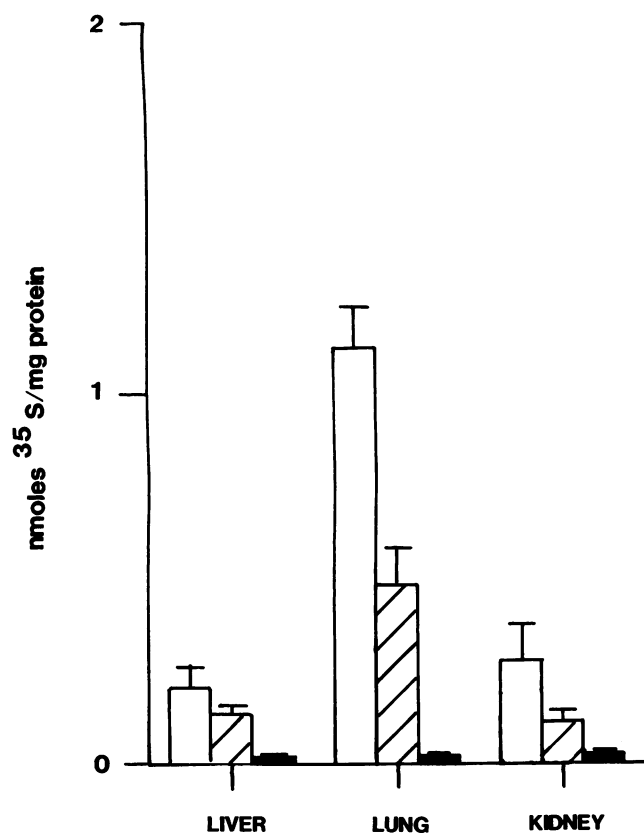
FIGURE 3.   The binding *in vivo* of ^{35}S derived from ^{35}S-thiourea to protein in lung, liver, and kidney. Open bars represent three normal rats challenged with ^{35}S-thiourea (15 mg/kg). Single hatched bars represent three resistant rats challenged with ^{35}S-thiourea (15 mg/kg). Closed bars represent binding that occurs during the induction of resistance (three dosed at 0.5 mg/kg for 3 days). Error bars represent standard deviation.

FIGURE 4.   Correlation between pulmonary edema (expressed as lung wet wt/body wt ratio) and *in vivo* binding of ^{35}S derived from ^{35}S-thiourea (15 mg/kg body wt, IP) to lung protein; (△), normal rats; (▲), resistant rats. Correlation coefficient = 0.963. (For experimental details see text.)

edema. In spite of the increased vesicular activity in the mesothelial cells, this effect of thioureas may not be due entirely to direct action of the toxin but a normal defense response to the presence of excess fluid in the pleural cavity.

Metabolism and binding studies with ^{14}C-thiourea suggested the need for metabolic activation of the toxin to a reactive metabolite that alkylates tissue proteins (*13–17*). The present studies demonstrate that protein binding of ^{35}S derived from ^{35}S-thiourea is very closely correlated with toxicity. Furthermore, importantly, they demonstrate that in resistant animals there is a dramatic reduction in bound [35]S. It is possible, therefore, that ^{35}S binding is a direct measure of toxicity and that the active metabolites alkylate tissue proteins causing the pulmonary edema that is typical of thiourea toxicity.

The identity of the active metabolite(s) of thiourea is unknown, but it has been suggested that ANTU can be activated to atomic sulphur (*12*). That a similar mechanism may operate for thiourea is supported by the inhibitory effects of ANTU and PTU on ^{35}S binding from
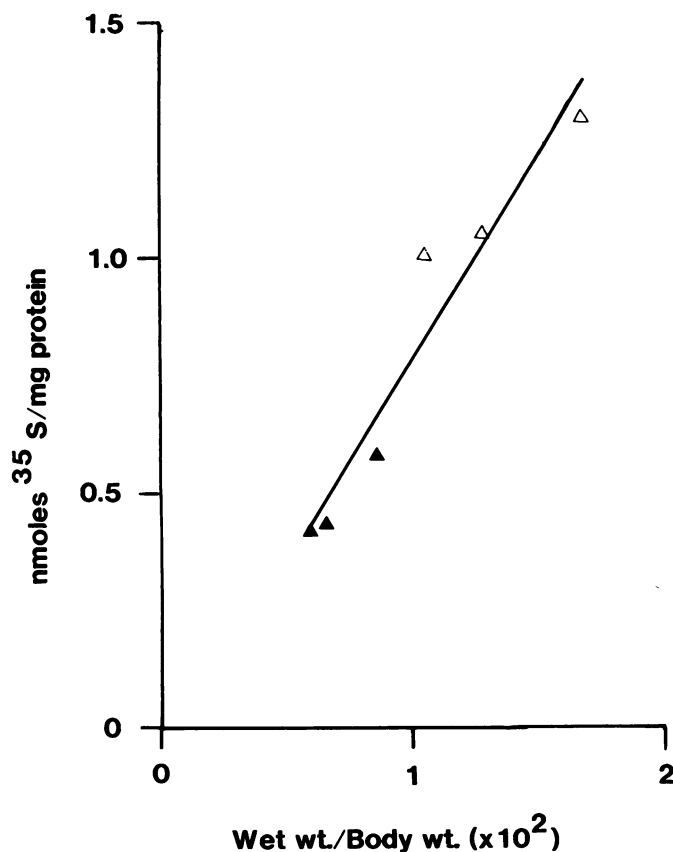
[35]S-thiourea *in vitro*. The binding of ^{35}S from thiourea to lung proteins *in vitro* and the decreased binding in lung slices from resistant rats suggests that activation takes place in the lung and that activated metabolites produced in the liver are not transported in the blood as, for example, pyrrolizidine alkaloids (*22*).

The number of proteins/enzymes reacting with metabolites of thioureas has yet to be established, although it is known that ANTU decreases the activity of cytochrome P-450 in lung and liver *in vitro* (*11,12*). However, activation of thioureas may also occur by non-microsomal enzymes because in lungs exposed to ^{14}C-thiourea *in vivo*, 75% of total ^{14}C bound to protein occurs in the supernatant fraction (*13,14*).

The mechanism(s) by which rats become resistant to thioureas following the administration of small nonlethal doses is intriguing. Unpublished work from these laboratories has shown that in anesthetized rats (with ureter cannula) there is no significant difference in the metabolism and rate of excretion of ^{35}S-thiourea between normal and resistant rats. Moreover, the differential bind-
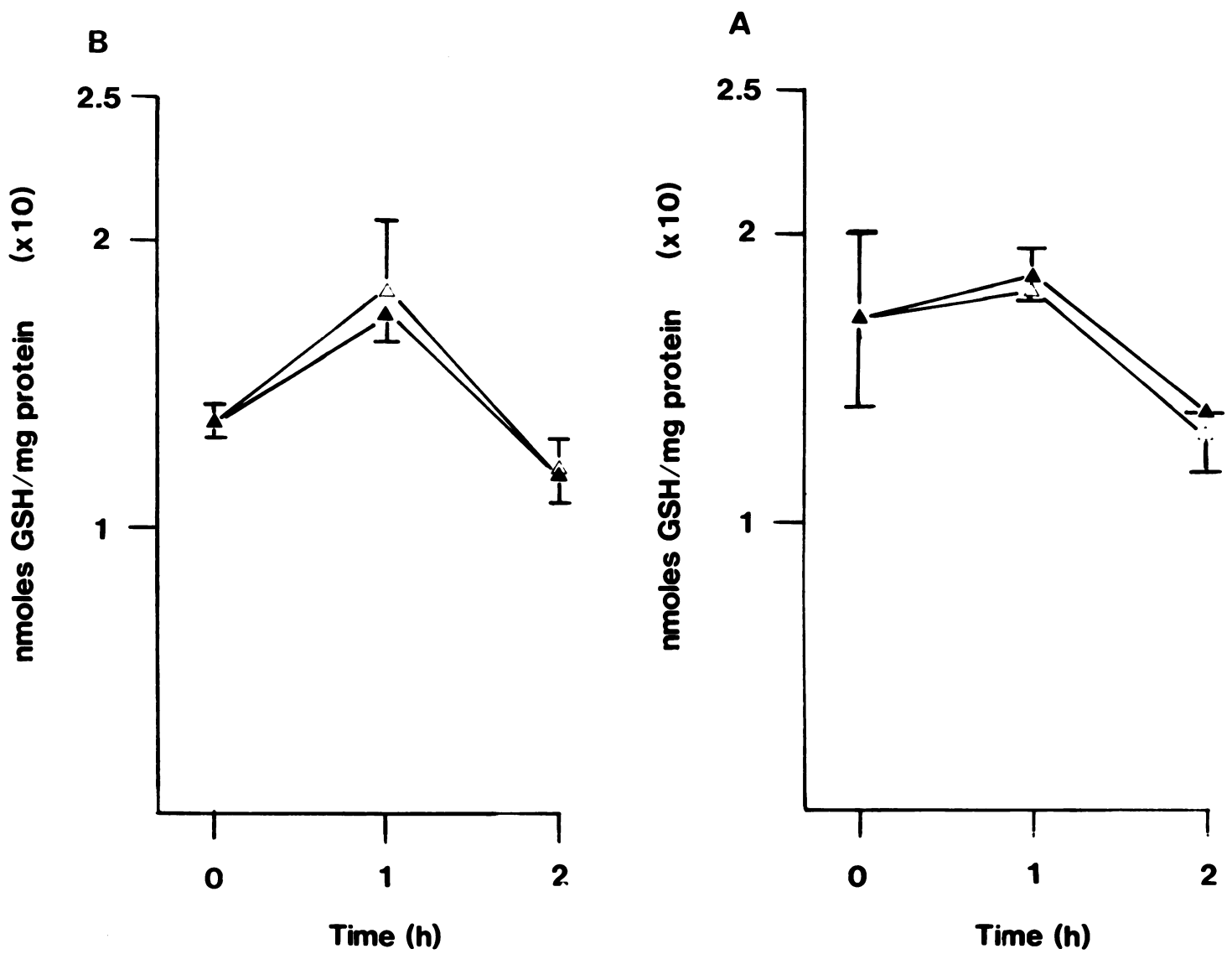
FIGURE 5. Glutathione levels in normal (A) and resistant (B) lung slices following thiourea exposure (1 mM) *in vitro*, n = 2. ($\triangle$), control incubations; ($\blacktriangle$), 1 mM thiourea. (For experimental details see text.)

ing to lung proteins *in vitro* from normal and resistant rats also suggests that resistance is afforded by biochemical alterations in lung itself and not by changes in the disposition of thiourea *in vivo*.

ANTU and PTU, which are edemagenic agents in their own right, compete with ^{35}S-thiourea for binding *in vitro*, suggesting that they act by the same pathway (Table 1). Interestingly, ANTU and PTU competed less well for the binding in resistant lung slices; this might suggest the presence of two types of binding site, a nonspecific site present in both normal and resistant lung slices, and a specific site(s) that is reduced or masked in resistant rats. These findings are in keeping with the two types of binding sites observed for ^{14}C-thiourea (15). The fact that urea does not compete for binding suggests that the C = S moiety is required for binding and toxicity.

Hollinger et al. (17) have reported that thiourea can deplete tissue glutathione by as much as 90% after 1 hr, and these data are consistent with the observations that depletion of tissue glutathione with diethylmalate leads to increased toxicity of thioureas, whereas coadministration with thiols prevents edema (18). It was surprising, therefore, that in the present study there was no evidence to suggest that thiourea is able to deplete lung glutathione levels *in vivo*. Indeed, at 2 hr after thiourea treatment, lung glutathione levels were increased by approximately 60%, at which point lung wet weight had increased by approximately 100%. The apparent difference between these results and those of Hollinger et al. (17) may be explained by the different assay systems for glutathione. Hollinger et al. employed the assay described by Ellman (23) that is nonspecific and measures total thiol. In this laboratory a fluorometric assay specific for glutathione was used (21). In addition it was shown that thiourea does not interfere with the latter assay, even when in a 10,000-fold excess (unpublished observations). Thus, in this study using a more specific assay for glutathione, there was no evidence to suggest that the appearance of edema coincided with a decrease in total glutathione levels in lung. Moreover, the induction of resistance cannot be explained by an increase in total lung glutathione because levels were not elevated in resistant rats.

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