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THE ROLE OF THE SODIUM-POTASSIUM ATPase DURING THE RESOLUTION OF PULMONARY EDEMA

by

Danny J. Zuege

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled "The Role of the Sodium-Potassium ATPase During the Resolution of Pulmonary Edema" submitted by Danny J. Zuege in partial fulfillment of the requirements for the degree of Master of Science.

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ABSTRACT

Previous studies have suggested that active sodium transport may be an important mechanism for the recovery from pulmonary edema. This mechanism for edema clearance has not been adequately studied using a realistic *in vivo* model of lung edema. The goal of these studies was to demonstrate the activation of lung sodium-potassium adenosine triphosphatase (Na-K-ATPase), an indirect marker of active sodium transport, during the recovery from an *in vivo* model of high permeability pulmonary edema. Lung Na-K-ATPase was activated during the recovery from thiourea-induced pulmonary edema. This activation could be reproduced by simple alveolar flooding. One mechanism for lung Na-K-ATPase activation was via an increase in the amount of Na-K-ATPase in the lung. Alveolar inflammatory cells did not contribute significantly to these changes. These results support the hypothesis that active sodium transport is operative during the process of recovery from pulmonary edema.

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CHAPTER 1

INTRODUCTION AND BACKGROUND INFORMATION

1.1 INTRODUCTION

Pulmonary edema, an abnormal, excessive collection of extravascular lung fluid, is a frequent and often fatal disease. In Canada, pulmonary edema associated with left ventricular failure accounted for 11322 hospital admissions for the year 1988-89 (1). This figure does not include the vast number of cases of pulmonary edema which complicate other primary disease processes. The risk of established pulmonary edema - the adult respiratory distress syndrome (ARDS) - is approximately 40% in the setting of systemic sepsis and has a mortality exceeding 50%, as well as substantial morbidity (2,3). Despite 20 years of progress in supportive care, there has been virtually no change in these high mortality rates for ARDS, with recently reported values still ranging from 41-74% (4).

Much progress has been made in the understanding of the normal physiologic processes that influence lung fluid balance and the pathophysiologic processes that lead to pulmonary edema. However, there has been relatively little research into the factors important to the resolution of pulmonary edema, factors critical to the potential treatment of this condition once established. Recently, Matthay *et al* (5) have reported that in patients being ventilated for pulmonary edema, clinical improvement was clearly related to

the ability of the lung to clear liquid, likely via active ion transport across an intact alveolar epithelial barrier. Furthermore, this ability to clear lung liquid was a good prognostic indicator for survival in prospectively studied ARDS patients (5). This information supports the concept that active lung liquid clearance may play an important role in the resolution of pulmonary edema in humans. As well, these findings justify more extensive in vivo confirmatory studies, as well as further investigation into the mechanisms underlying active ion transport and lung liquid clearance in pathologic states of pulmonary edema. A better understanding of these mechanisms and ways to accelerate lung liquid clearance may lead to more rational and innovative approaches to therapy for this important clinical entity.

The overall objective of this project was to demonstrate that active sodium transport is operative during the resolution from pulmonary edema using a realistic in vivo model of lung injury. Active sodium transport was evaluated indirectly by monitoring the activity of pulmonary sodium-potassium stimulated adenosine triphosphatase (Na-K-ATPase), a ubiquitous sodium transporting enzyme.

1.2 BACKGROUND INFORMATION

1.2.1 Mechanisms of lung liquid clearance and sodium transport

When one is evaluating the factors regulating the resolution of pulmonary edema, one is really evaluating the mechanisms and regulation of lung liquid clearance. Unfortunately, data on lung liquid clearance in humans, or even in the setting of pathologic states of pulmonary edema, is very limited; therefore, much of the present understanding of lung fluid balance is derived from non-edematous or flooded animal lung preparations.

Older theories on lung fluid balance focussed primarily on Starling forces and assumed that the alveolar epithelium was a passive barrier. It was discovered that the alveolar epithelium had a very low permeability to hydrophilic molecules (6-8) and formed a high resistance epithelium, with an estimated in vivo resistance of 18000 Ohm/cm^2 (9). This was in comparison to the pulmonary vascular endothelium, which was comparatively permeable to solutes (7,8).

Matthay et al (10,11) utilized an alveolar flooding model in sheep, instilling autologous serum into the airways, and demonstrated that liquid clearance was much faster than protein clearance. Therefore, the protein in the remaining alveolar fluid became more concentrated with time creating a progressively unfavorable osmotic gradient

for liquid absorption. However, lung liquid resorption continued; and thus, could not be explained by osmotic forces alone. This concept was supported by experiments done by Effros et al (12) which also showed that lung sodium transport continued in the presence of an unfavorable osmotic gradient across the alveolus. Hydrostatic forces could not be invoked to explain the observed fluid clearance either because, first, there were no significant changes in left atrial or pulmonary vascular pressures in the above studies (10,11), and second, both measured and estimated values for pulmonary perimicrovascular pressure have been too low to overcome the osmotic gradients created during alveolar liquid clearance (13-15). As a result of the above findings, active transport processes were then considered as possible mechanisms to account for the observed clearance of lung liquid (10,11).

Although Starling forces are presently considered to be less significant than active transport processes in the resolution of pulmonary edema, they are important in the induction of pulmonary edema (16). This is seen clinically in situations of high pulmonary venous pressure, such as left ventricular failure, or in situations of low colloidal osmotic pressure, such as hypoalbuminemia, which, when induced acutely, can contribute to the creation of pulmonary edema (17).

The presently accepted hypotheses for mechanisms of lung liquid clearance focus on active sodium transport, with water subsequently following passively along the newly created osmotic gradients. The evidence for this comes from studies of whole animals (18,19), isolated perfused rat lungs (9,20,21), and isolated or cultured monolayers of alveolar type II cells (AIIC) (22-24).

Berthiaume et al (18) utilized an alveolar flooding model in anesthetized, ventilated sheep in which autologous serum with or without the beta agonist terbutaline (10^{-5} M) was instilled into the lung. Treatment with terbutaline resulted in an increase in lung liquid clearance that could not be explained by changes in hemodynamics or lung lymph flow. The terbutaline effect was virtually completely inhibited by including the sodium transport inhibitor amiloride (10^{-3} M) in the instillate, indicating the dependance of the beta-adrenergic effect on sodium transport. Smedira et al (19) similarly evaluated lung liquid clearance in anesthetized adult rabbits and demonstrated that amiloride (10^{-4} M) inhibited 75% of the basal fluid clearance. However, there was no response to beta-agonist therapy, indicating species differences in this effect. Thus, whole animal studies demonstrate a pathway for lung liquid clearance that is amiloride inhibitable, which suggests that it is dependent on sodium transport.

Complementing whole animal studies are several investigations which utilize isolated, perfused lungs derived from adult rats. Goodman et al (20) monitored unidirectional (alveolus-vascular) (^{22}Na) fluxes to derive permeability-surface area products for sodium (PS-Na), thus avoiding the problems with estimation of total alveolar surface area. The inclusion of either amiloride (10^{-3} M) in the instillate or ouabain ($5x10^{-5}$ M), a specific inhibitor of the Na-K-ATPase, in the perfusate, reduced the PS-Na by 30% from basal values. Adding terbutaline ($2.4x10^{-5}$ M) to the perfusate increased the PS-Na by 28%. The beta-adrenergic effect was again amiloride inhibitable suggesting that it was sodium dependant. Since the PS-Na includes both active and passive sodium fluxes, one cannot differentiate between these two modes of transport, although PS values for (^{14}C)-sucrose, included as a marker for non-active transport, were not affected by any of the manipulations. Basset et al (9,21), using a similar model, also showed that fluid reabsorption was inhibited by including ouabain in the perfusate or by including amiloride in the instillate. When a sodium-free instillate was used, there was no liquid clearance. Alveolar amiloride in combination with either phloridzin (10^{-3} M), an inhibitor of the sodium-D-glucose symport (25), or a glucose-free instillate, completely inhibited liquid clearance. Phloretin, an inhibitor of non-sodium dependant facilitated glucose transport (26), had no

effect. In contrast, O'Brodovich et al (27) found phloridzin had no effect on lung water clearance using an in vivo neonatal guinea pig model. The differing results may be due to the different stages of lung development or animal species used in the two models. In the studies by Basset et al (9,21), unidirectional (alveolar-vascular) (^{22}Na) fluxes were inhibited 55% by the presence of both amiloride and phloridzin, suggesting that 45% of the basal unidirectional sodium flux could depend on paracellular movement of sodium. Thus, studies of isolated rat lungs suggest apical (alveolar) mechanisms for sodium entry and a basally (interstitially) located ouabain-sensitive sodium pump. Beta-adrenergic stimuli also produce an amiloride inhibitable (sodium dependant) increase in lung liquid clearance as demonstrated in whole animal studies. However, these studies also show the presence of a passive, paracellular pathway for sodium absorption that may account for a substantial proportion of basal sodium movement.

Although the aforementioned experimental models have used amiloride to demonstrate the sodium dependence of lung liquid clearance, they cannot address by which cellular mechanism sodium transport occurs. This is because amiloride inhibits sodium transport via 2 mechanisms: at low concentrations (10^{-6} M) amiloride inhibits sodium channels; at higher concentrations (10^{-3} M), amiloride also inhibits the sodium-hydrogen ion antiport (28). As used in

the above studies, one cannot differentiate as to which of these apical sodium transport mechanisms may be operative as high (10^{-3} M) doses were used. Further, as amiloride is significantly bound by protein (29), the concentration of the free (active) drug in the protein-containing instillates used in the above studies is uncertain. Thus, one cannot utilize the concentration of amiloride in these instillates to determine mechanism of action.

A common feature of the above studies is that they utilize artificially created alveolar flooding as a model to examine lung liquid clearance. Although useful experimentally, this model is a relatively unrealistic simulation of the human condition of pulmonary edema, particularly with regard to edema secondary to an increase in the permeability of the alveo-capillary barrier. In such situations there is often epithelial cell damage, and perhaps dysfunction, induced by various circulating cells and mediators (interleukins, endotoxins, tumor necrosis factors, complement etc.). Thus, mechanisms identified in models of artificially induced alveolar flooding should be confirmed in more realistic models of pulmonary edema wherever possible.

On a cellular level, ion transport in the alveolar epithelium has principally been studied utilizing isolated and cultured monolayers of AIIC, as there is a well identified technique for their isolation and culture.

Unfortunately, there is, as of yet, no technique for the isolation of the principle component of the alveolus, in terms of surface area contribution - the type I cell. Clara cells have recently been identified as possessing active solute transport properties (30,31), as have distal bronchi and bronchioli (32,33); however, the alveolus is favored to be the primary site of liquid absorption as the alveolar epithelium has by far the largest surface area available for this process (34).

For the study of bioelectric properties, AIIC have been cultured to monolayers by several laboratories (35). Whether or not many early attempted monolayers were truly representative of functional epithelia in vivo is questionable however, given that the transepithelial resistances measured for these early monolayers were <300 Ohm/cm^2 (23). These are far below those estimated in vivo (about 18000 Ohm/cm^2)(9), or those found with newer cultures on treated Nucleopore filters (>2000 Ohm/cm^2) (24,36). Distinction of cellular versus paracellular ion flux, thus, may have been inaccurate in older monolayer preparations. Goodman et al (22) cultured AIIC to monolayers on non-porous media and demonstrated the formation of fluid-filled domes. These domes are generally thought to arise secondary to active solute transport from the medium above the monolayer to the substratum, with water following passively (37). Dome formation was dependant on sodium in the medium and was

inhibited by ouabain (10^{-3} M) or amiloride (10^{-3} M), with no effect of inhibitors of chloride transport. High resistance AIIC monolayers examined in Ussing chambers have been demonstrated to maintain a transepithelial potential difference of 9.7 mV, apical side negative (36). In the absence of any osmotic, ionic, chemical, or electrical gradients, these monolayers produce an electrical current (short-circuit current) that is by exclusion attributable to active ion transport (24,36). This current was inhibited 68% and 91% by application of apical amiloride and basal ouabain, respectively, and stimulated 97% by terbutaline. Finally, bidirectional sodium flux measurements, using high resistance monolayers, indicate a net basal sodium flux, apical to basal direction, in the absence of any gradients, that was again inhibited by apical amiloride or basal ouabain and stimulated with basally applied terbutaline (24). Thus, in vitro preparations of AIIC monolayers clearly have sodium transport capabilities, the general characteristics of which resemble those found in whole animal and isolated lung studies.

Other apical ion transport mechanisms identified in AIIC include the aforementioned sodium-glucose cotransport (9,21,27), sodium-hydrogen ion exchange (38,39), sodium-amino acid cotransport (40), and sodium-conducting ion channels (41-43). The conflicting data on the relative importance of sodium-glucose cotransport to apical sodium

absorption has been discussed (9,21,27). Sodium-hydrogen ion exchange, although functional in AIIC, has recently been shown to contribute relatively little to net sodium transport in both fetal (44) and adult (45) AIIC monolayer preparations as well as in an in vivo neonatal guinea pig preparation (27) using relatively specific inhibitors of this transport mechanism. Sodium-amino acid cotransport most likely serves a nutritive function (46), its role in lung liquid clearance being presently unknown. Finally, sodium channels have been directly demonstrated on AIIC using fluorescent polyclonal antibodies (43). Investigations aimed at physiologic demonstration of sodium channels on AIIC via patch-clamping, however, have demonstrated cation channels which were either equally permeable to sodium and potassium (41,42) or moderately more permeable to sodium over potassium (43). Thus, at least two candidate mechanisms for apical sodium entry into AIIC have been identified so far - sodium channels and sodium-glucose cotransport. The Na-K-ATPase, however, is the only mechanism for basal sodium extrusion so far identified.

There are several limitations to drawing conclusions on lung liquid transport via the evaluation of isolated AIIC alone. Firstly, the majority of alveolar surface area is not studied, as AIIC comprise only 3.8% of the total apical alveolar surface area in rats (47), and 7.1% of the same area in humans (48). Secondly, the recovery of AIIC from

intact lungs is only 10-20% (35,49). Whether these isolated cells are representative of the 80-90% of AIIC which are not successfully isolated is not certain. Thirdly, there is a tendency for AIIC in culture to undergo physiologic and morphologic changes with time (35,49-54), which has been suggested to represent de-differentiation to more prototypal cell types (51). Isolation of AIIC has been demonstrated to induce a cell cycle block relative to AIIC in vivo (52), as well as significantly inhibiting total protein and surfactant lipid and apoprotein mRNA synthesis (53). Morphologically, AIIC in culture transform from a cubiodal to a more flattened shape, and gradually lose their characteristic lamellar bodies (49). These findings raise the question of whether or not these cultured cells functionally represent AIIC in vivo. Finally, a limitation to the study of isolated cells in general is that many of the components of the physiologic mileau, particularly hormonal influences, are absent, thus limiting the generalizability of in vitro studies to in vivo situations. A complete understanding of the physiology of alveolar sodium transport will, thus, likely require a correlation between in vitro and in vivo observations.

With the above information, one can illustrate the possible mechanisms of sodium transport, although limited primarily to the AIIC, in a visual manner (figure 1.1).

Inhibitors:

1. Ouabain 10^{-3} M
2. Amiloride 10^{-6} M
3. Amiloride 10^{-3} M
4. Phloridzin 10^{-3} M

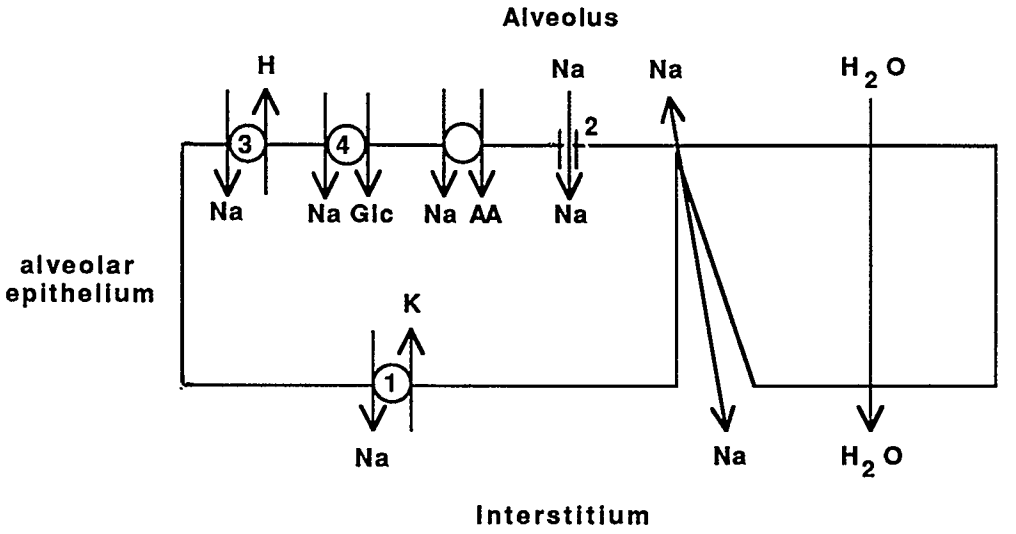


Figure 1.1 Theoretical model for sodium transport in AIIC epithelium

Here, the cellular pathway involves several potential mechanisms for apical (alveolar) sodium entry and a basolateral mechanism for intracellular sodium extrusion into the interstitium. A paracellular pathway for sodium movement is also illustrated. In this case, sodium would follow its ionic gradient, the ion flow being limited by the low passive sodium permeability of the epithelium. Lung water clearance is, thus, secondary to active sodium

transport in this model. Although all the transport mechanisms discovered with in vivo preparations can be found in the AIIC, the contributions of the remaining alveolar components await improved cell isolation and culture techniques. Until these techniques are developed, conclusions concerning mechanisms of whole lung sodium and liquid clearance should include confirmation via whole lung studies.

1.2.2 Function and regulation of the Na-K-ATPase

1.2.2.1 General characteristics of the Na-K-ATPase

The Na-K-ATPase is a ubiquitous sodium and potassium transporting enzyme found in virtually all plasma membranes of the body (55). It is generally located on basolateral portions of epithelia although, in brain choroid plexus (56) and retinal pigment epithelium (57), it is found apically. The complex reaction cycle involves at least two conformational states to transport 3 sodium molecules out of cells and 2 potassium molecules in, with energy being derived from ATP hydrolysis (58). Necessary for activity are potassium and sodium ions, ATP, magnesium ions (required for ATP hydrolysis), and phospholipid. The cardiac glycoside ouabain, a specific inhibitor of Na-K-ATPase, acts on the extracellular side of the membrane to block potassium-dependant dephosphorylation (58,113). The active enzyme contains two subunits labelled alpha and beta (55). The alpha subunit (theoretical M_r = 110 Kda) has the binding

sites for sodium, potassium, ouabain, and ATP, whereas the heavily glycosylated beta subunit (M_r = 35-55 Kda) is of unknown function, although absolutely necessary for enzyme activity (55,59). There are 3 identified isoforms of the alpha subunit, designated alpha-1,2,3, that are distinguished by monoclonal antibodies, differing DNA and protein sequences, and varying ouabain sensitivities. The Kd for ouabain ranges from 10^{-5} - 10^{-4} M for the alpha-1 isoform as compared to 10^{-8} - 10^{-7} M for the alpha-2 and 3 isoforms (60,61). These isoforms are distributed in a tissue specific manner, with alpha-1 being found in virtually all tissues, and being the only form found in the lung (55,62). The alpha-2 isoform is restricted to skeletal and cardiac muscle, and the alpha-3 isoform is found primarily in the brain (55,62,63). The beta subunit has also recently been discovered to consist of at least 3 isoforms, with the beta-2 isoform being identified as a glial adhesion protein in astrocytes (61,64).

1.2.2.2 Regulation of Na-K-ATPase activity

There has been a plethora of research into the regulation of the Na-K-ATPase. On reviewing this subject two points become clear. First, very rarely are the effects of agents on Na-K-ATPase activity distinguished as primary (direct) versus secondary (indirect via various mechanisms, including second messengers, and especially changes in intracellular sodium concentration). Second, when

interpreting tissue-specific regulatory mechanisms, it would seem prudent to compare tissues with identical alpha subunit isoforms; thus, lung Na-K-ATPase may be best represented by other alpha-1-isoform-containing tissues like kidney.

The dominant short-term regulator of Na-K-ATPase activity is the intracellular concentration of sodium, with increasing concentrations rapidly leading to saturable increases in activity (65-67). As well, changes in intracellular sodium concentration are major short-term regulatory mechanisms for many extracellular signals (67,68).

Activators of Na-K-ATPase include catecholamines, of which beta agonists are of particular interest in the lung as they have been demonstrated to increase lung liquid clearance in sheep (18) and fetal lambs (69), all in an amiloride inhibitable manner. Beta agonists bind to specific membrane receptors and are thought to act via stimulation of adenylate cyclase resulting in increases in intracellular cAMP concentrations (70). Cellular effects are tissue specific and may depend on the particular cell's signal transduction mechanism. For example, a cAMP dependant protein kinase (PKA) may phosphorylate various proteins, one of which has been demonstrated to be the Na-K-ATPase (70,71-73).

Antidiuretic hormone (ADH, vasopressin) also acts via the activation of the adenylate cyclase/cAMP pathway, and

has been shown to enhance sodium transport in epithelia (amphibian skin and bladder) (68). The short-term effect of ADH is currently thought to involve an increase in apical sodium entry with a secondary increase in Na-K-ATPase activity. A more sustained, long-term effect is also postulated that involves new pump synthesis (68,74). In contrast to the likely PKA dependant action of catecholamines, the cellular effects of ADH may involve dephosphorylation of membrane proteins, implicating the action of cAMP dependant phosphatases (70).

Studies examining the direct effects of cAMP on Na-K-ATPase have yielded conflicting results, which may be the result of tissue-specific signal transduction pathways (eg. PKA versus cAMP dependant phosphatases). In pancreatic islets, exogenous cAMP and phosphodiesterase inhibitors (theophylline/caffiene) inhibited Na-K-ATPase (75), whereas in kidney, the above agents stimulated activity in renal cortex but inhibited activity in renal medulla (76). These effects are of interest as both cAMP analogues and phosphodiesterase inhibitors have stimulated lung liquid clearance in isolated whole lung preparations (77,78), and the combination of the two agents was effective in stimulating liquid clearance in intact sheep (79). Although the mechanism stimulating sodium transport in these studies is uncertain, possibilities include an increase in apical sodium channel conductance, an increase in the number of

sodium channels, an increase in apical sodium entry via other transport mechanisms (eg. sodium-glucose cotransport), or a direct or indirect increase in Na-K-ATPase activity.

It is well known that adrenalectomized rats have tremendously decreased renal Na-K-ATPase activities which are completely normalized by mineralocorticoid (eg. aldosterone) administration (68). Aldosterone induces synthesis of several proteins, one of which has been identified as the Na-K-ATPase (68,74). Aldosterone is thought to have a short-term effect, seen after <1 hour of exposure, of increasing amiloride-inhibitable cellular sodium entry, with secondary activation of Na-K-ATPase (68). A long-term effect is also observed, after at least 2-3 hours of exposure, of increasing Na-K-ATPase activity by inducing new pump synthesis (68). Barlet-Bas et al (80), based on studies on intact renal collecting duct, postulate that mineralocorticoids may regulate the size of a latent pool of Na-K-ATPase, not associated with the plasma membrane, that is recruited during short-term regulation of the enzyme by changes in intracellular sodium. Two studies (81,82) exposed isolated Xenopus laevis pulmonary epithelium to aldosterone (10^{-6} M) and demonstrated that, after a one hour lag period, there was an increase in transepithelial sodium current which reached a maximum of about twice baseline after 4-5 hours. Oxytocin, in combination with aldosterone, had a synergistic effect on sodium transport.

As well, lungs isolated from rats previously aerosolized with aldosterone have recently been shown to exhibit increased sodium and water clearance relative to untreated animals (83). Thus, pulmonary epithelia seem to be aldosterone responsive in at least two species. Glucocorticoids have also been demonstrated to partially restore renal Na-K-ATPase activity in adrenalectomized rats by inducing new pump synthesis (68,74).

Other stimulators of Na-K-ATPase activity include thyroid hormones and insulin. Thyroid hormones result in an increase in Na-K-ATPase mRNA and protein synthesis to result in an increase in cellular activity, however, often do so in a discordant manner (68). In rat heart, thyroid hormone treatment resulted in 9 and 22 fold increases in Na-K-ATPase alpha and beta subunit mRNA transcripts respectively, however, induced only a 2 fold increase in enzyme activity. This strongly implicates translational and/or post-translational modulatory mechanisms in the thyroid hormone effects on Na-K-ATPase (68,85). Insulin is also known to increase Na-K-ATPase activity, likely via altering the sodium affinity of the Na-K-ATPase (68).

Inhibitors of Na-K-ATPase activity include calcium, where in a rat myometrial microsomal fraction, 3 uM free calcium inhibited 45% of basal enzyme activity, an effect which was dependant on the presence of functional PKA (86). Activation of protein kinase C (PKC), either directly with

diacylglycerols or phorbol esters (87) or indirectly with dopamine (87-89) in rat renal proximal tubule, inhibited Na-K-ATPase activity. Direct phosphorylation of Na-K-ATPase by PKC has also been shown to inhibit enzyme activity (73). Sapijaszko et al (90) have recently shown that PKC is activated late in the recovery from alveolar flooding in the rat, at a point when all liquid has been cleared from the alveolar space. This is suggestive of an inhibitory role for PKC on liquid clearance, perhaps terminating the active sodium transport process once excess fluid has been resorbed. Finally, endogenous prostaglandins have been shown to have a basal suppressive effect on renal collecting tubule Na-K-ATPase activity (91).

The described potential regulators of Na-K-ATPase are illustrated in figure 1.2.

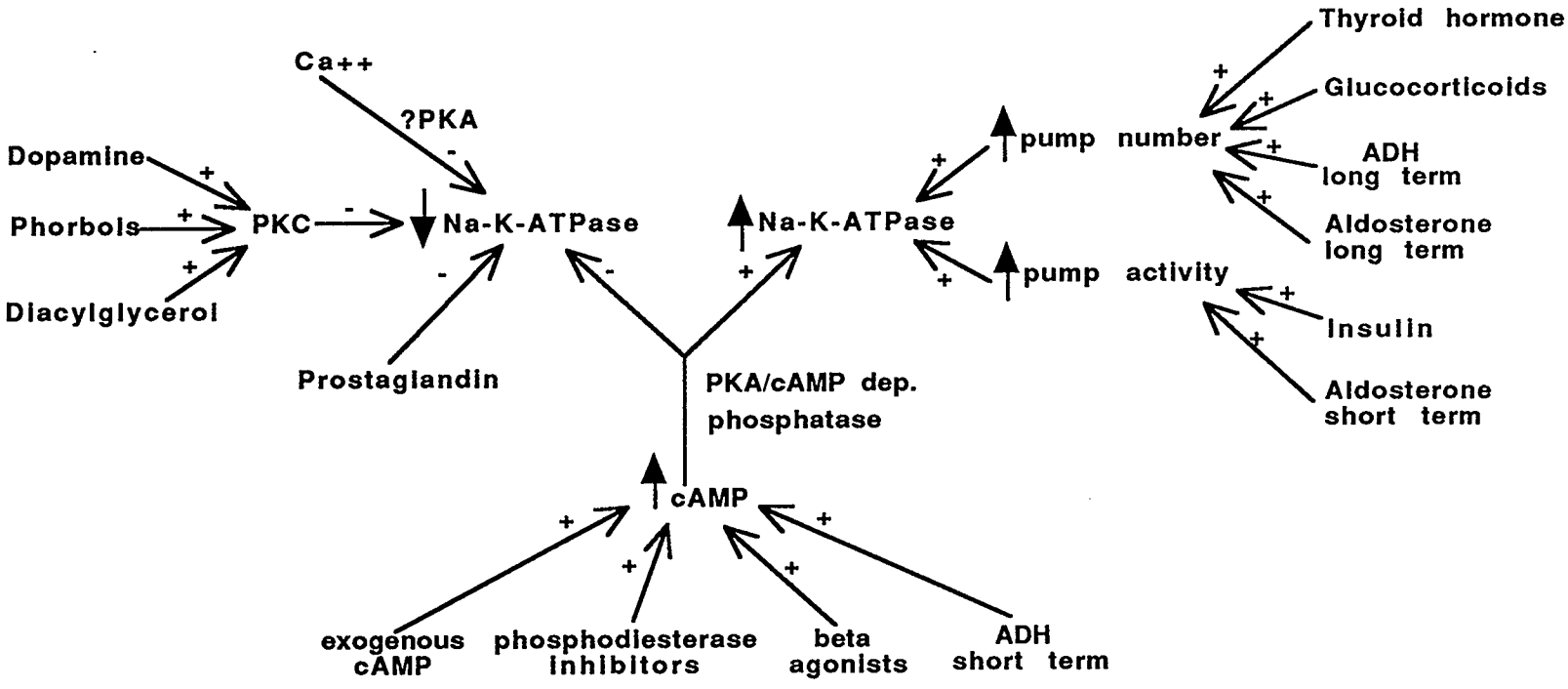


Figure 1.2 Some of the potential intra- and extracellular regulators of Na-K-ATPase.

1.2.3 Na-K-ATPase in lung

Direct studies of Na-K-ATPase in lung tissue have been few, and there has been only one study investigating its role in the resolution of pulmonary edema. It has been demonstrated, using cDNA probes specific for Na-K-ATPase alpha subunit mRNA, that the alpha-1 isoform is the predominant subunit present in lung tissue (63). Alpha-1 mRNA increases in abundance during neonatal development, consistent with new protein synthesis to reach adult levels of enzyme, and is maintained at low levels during normal adult life (63).

Sheenberger et al (92) used a monoclonal antibody to partially purified rat kidney Na-K-ATPase to demonstrate that specific binding to the enzyme in the alveolus was limited primarily to the basolateral aspects of the AIIC. Although there was no binding over background levels to any other component of the alveolar epithelium, the often stated conclusion that only AIIC contribute significantly to total alveolar Na-K-ATPase activity is not valid. First, this study has never been repeated, specifically with other high affinity isoform-specific antibodies (55). Second, given that the results of the above study suggest that AIIC **may** have a **relatively** higher concentration of membrane bound Na-K-ATPase, the fact that AIIC comprise only 3.8% of the total apical alveolar surface area in rats (47), and 7.1 % of the corresponding area in humans (48), means that even if the

other components of the alveolar membrane had only one tenth the total cellular Na-K-ATPase, given that their surface area contribution is so high, the proportion of Na-K-ATPase contributed by the AIIC would be only 28.3% of the total alveolar Na-K-ATPase. Cells with only one tenth the total cellular Na-K-ATPase of AIIC conceivably may not result in specific binding over that of background - in the study by Sheenberger et al (92), 52% of AIIC seen histologically showed no binding of Na-K-ATPase specific antibody. Thus, the non-type II cell components of the alveolar membrane clearly could contribute significantly to alveolar sodium transport.

Recently, Chapman et al (93) utilized the uptake of (^{86}Rb), which acts as a potassium analogue, as a physiological measure of Na-K-ATPase activity, and tritiated ouabain binding to quantitate the number of enzyme molecules per cell, in isolated fetal and neonatal AIIC. It was shown that perinatally, the total cellular activity of Na-K-ATPase increases secondary to an increase in specific molecular activity, whereas during postnatal development, the total activity increases secondary to an increase in enzyme number. Thus, this study identifies two general mechanisms by which lung Na-K-ATPase activity may be regulated: via changes in the quantity of enzyme or changes in the molecular activity of the enzyme.

In the only study examining lung Na-K-ATPase in the setting of lung injury, Nici et al (94), exposed rats to 60 hours of 100% oxygen, followed by up to 10 days of recovery. They demonstrated increases in lung Na-K-ATPase alpha and beta subunit mRNA expression, detectable after 60 hours of hyperoxic exposure, and increased lung Na-K-ATPase protein expression, detectable by 1 day of recovery. It is difficult to interpret the meaning of these results, however, as both Crapo et al (47) and Thet et al (95), using similar hyperoxic exposure in rats, found that by 60 hours of oxygen exposure there were significant increases in the number of alveolar type II, and especially, interstitial cells. By 3 days, of recovery, Thet et al (95) found further increases in alveolar type II and interstitial cells and increased numbers of endothelial cells. Thus, whether the increase in mRNA and protein expression observed in the study by Nici et al represented a cellular response to the edema or simply a proliferative response to the injuring agent is uncertain. As well, it remains unknown as to whether the changes observed by Nici et al would translate into an increase in overall enzyme activity or sodium transport, especially considering the extensive epithelial damage produced in this model of lung injury (47,95).

1.3 SUMMARY

In summary, lung liquid clearance has been shown in numerous studies to be dependent on the active transport of

sodium by the alveo-capillary barrier. The active nature of this process has been confirmed in a single study in humans (5). The major components of active sodium transport in the alveolar epithelium include several potential mechanisms for apical sodium entry and a basolaterally located Na-K-ATPase for the active extrusion of sodium into the interstitium, with water following passively. Several potential regulatory factors in the lung have been identified for this process, notably beta-agonists, cAMP, aldosterone, and PKC. The Na-K-ATPase is the 'active' (ATP requiring) component of the pulmonary sodium transport process.

There have been several studies which have demonstrated the importance of active sodium transport during the recovery from artificially induced alveolar flooding using ex vivo isolated lung and in vivo whole animal preparations. In contrast, there has been relatively little study of active sodium transport during the resolution from realistic in vivo models of pulmonary edema. The overall goal of these studies was to demonstrate the stimulation of active sodium transport during the resolution from such a realistic model of pulmonary edema. As transepithelial sodium fluxes cannot be monitored in vivo, another marker of active sodium transport was required. Given that the Na-K-ATPase is the only known mechanism for basolateral extrusion of sodium once absorbed from the apical alveolar membrane, the activity of this enzyme should represent a measurable index

of active sodium transport. The study by Nici et al (94) provides preliminary evidence suggesting a potential role for Na-K-ATPase regulation during the recovery from lung injury.

The signals for activation of lung sodium and liquid clearance in states of pulmonary edema remain unknown. Possibilities include hypoxemia, a consequence of impaired gas exchange, which can stimulate ADH release (96), the local action of inflammatory mediators, or neurally mediated signals consequent to pulmonary edema (97). Hormonal factors such as catecholamines and glucocorticoids, which are found in increased quantities in many critically ill patients (98,99), or aldosterone, which is found in increased quantities in patients with low circulating fluid volumes (98), have all been demonstrated to stimulate the Na-K-ATPase, and are other factors that may stimulate lung sodium, and therefore liquid, transport in patients with pulmonary edema.

In the setting of this preliminary information, the initial objective of this project was to examine the activity of lung Na-K-ATPase, an index of active sodium transport, during the recovery from a realistic in vivo model of pulmonary edema. A secondary objective of this project was to examine the mechanisms underlying any changes in lung Na-K-ATPase activity during edema recovery.

CHAPTER 2

GENERAL METHODS

The purpose of this chapter is to outline those methods common to several areas of this project. Methods specific to a chapter will be described in the *methods* section of that chapter.

2.1 Animals

All experiments in this project utilized specific 'pathogen-free', adult, male Sprague-Dawley rats (weight 275-450 grams) obtained from Charles River (St. Constant, Quebec). The rats were maintained in cages (2-3 rats/cage) in an air filtration unit so as to minimize airborne infection. The animals were kept in a 12 hour light-dark cycle, and given free access to tap water and standard rat feed (Purina). All protocols had approval of the University of Calgary animal review committee.

2.2 Determination of wet/dry ratio

The 'wet/dry ratio' provides a quantitative measure of lung water, and thus, was used in this study to estimate the magnitude of pulmonary edema. This value represents the ratio between extravascular lung water (Q_{wl}) and blood-free dry lung weight (dQl). Its determination follows the gravimetric method of Selinger *et al* (100). Briefly, lung tissue was homogenized (Janke and Kunkel Ultraturrax) with an equal mass of water, and then centrifuged (5000g x 60 minutes, $4^{o}C$) to yield a supernatant. At the time of

sacrifice prior to exsanguination, a sample of blood was taken from the abdominal vena cava into a heparinized syringe. Samples of the homogenate, supernatant, and blood were then dried at 37^{O}C for 2 days to obtain their fractional content of water. Hemoglobin concentrations of the supernatant and blood were determined using the cyanohemoglobin technique (101), with the ratio between the two used to estimate the mass of blood in the lung, so as to correct the calculations for Q_{wl} and dQl accordingly. All measurements were made in duplicate.

2.3 Protein quantitation

Protein concentration in lung supernatant preparations was measured by the method of Bradford (102), using bovine serum albumin as a standard.

2.4 DNA quantitation

DNA was measured via a modified diphenylamine assay as described (103,104). In brief, to identical series of volumes (20-100ul) of lung supernatant preparation and simultaneously run DNA standards (Calf thymus DNA (Sigma) - 0.25 mg/ml), 1 ml of cold 10% (w/v) trichloroacetic acid (TCA) was added and the tubes were allowed to sit at 4^{O}C for a minimum of 4 hours to precipitate DNA and protein. The tubes were then centrifuged at 2400g x 15 min. at 4^{O}C and the resulting supernatants were carefully aspirated and discarded. The pellets were resuspended in 0.5 ml of 5% (w/v) TCA, vortexed, and then incubated for 30 min. in a

90^{O}C water bath to hydrolyze the DNA. After cooling, 0.5 ml of freshly prepared chromogenic reagent (1.47% (w/v) diphenylamine, 0.01% (v/v) acetaldehyde) was added to each tube and the samples were incubated at 37^{O}C for a minimum of 12 hours to allow color development. The tubes were then centrifuged at 2400g x 15 min. at 20^{O}C to pellet the insoluble protein, and the absorbance of the supernatants was read at 600 nm on a spectrophotometer (Beckman DU62). The absorbances of the samples and standards were plotted against volume and slopes were obtained by linear regression analysis. The DNA concentration of the samples were then determined by multiplying the ratio of slopes between the samples and standards by the DNA concentration of the standards.

2.5 Statistics

Data are presented as the mean +/- standard error of the mean (SEM) throughout. The statistical analyses applied in specific experiments are outlined in individual methods sections. All p values are two-tailed and p values <0.05 were taken to indicate statistical significance.

CHAPTER 3

IMPLEMENTATION AND OPTIMIZATION OF Na-K-ATPase ASSAY METHODOLOGY FOR USE WITH LUNG SAMPLES

3.1 LUNG SUPERNATANT PREPARATION

3.1.1 Introduction

The initial step in this project was the implementation and optimization of an assay technique for the quantitation of Na-K-ATPase activity. The first task in this regard was to obtain a suitable sample of lung tissue for biochemical analysis. A suitable sample should fulfill several criteria:

1. The sample should contain virtually all of the lung Na-K-ATPase. This ensures that one is examining the entire enzyme sample, rather than a potentially unrepresentative subsample.
2. The sample should be entirely liquid in nature, so as to facilitate accurate volume measurement.
3. The sample should be homogeneous, so that repeated measurements on the same sample are always representative of the original extract.

The method chosen to obtain this sample followed the initial steps of plasma membrane purification protocols used by others in lung (105) and kidney (106) tissues. The lung was homogenized in an isotonic buffer followed by differential centrifugation to sediment debris. The resulting sample, hereby termed the 'lung supernatant preparation', fulfilled

the above criteria and was used in all subsequent steps of this project.

3.1.2 Methods

3.1.2.1 Lung supernatant preparation

The general sequence for this preparation is outlined in figure 3.1.

Normal male Sprague-Dawley rats were anesthetized with pentobarbital (60 mg/Kg intraperitoneal) and sacrificed by exsanguination via transection of the abdominal aorta and inferior vena cava. The chest was then carefully opened, the trachea clamped, and the lungs and mediastinal structures removed in toto. The smaller left lung was then transected close to the hilum, leaving the trachea and mainstem bronchus behind, and placed into a cold centrifuge tube. 10 ml of homogenizing buffer (sucrose 0.25M, EGTA 1mM, PMSF 1mM, leupeptin 25 ug/ml, DTT 1mM, imidazole 25 mM, pH 7.40) was then added and the mixture was homogenized at 24000 rpm in a rotor homogenizer (Janke and Kunkel Ultraturrax) for 3 x 20 second intervals on ice to minimize any heat buildup. The sample was then centrifuged at low speed (1500g x 15 min $4^{O}C$) to sediment connective tissue fragments, unbroken cells, and nuclei (105,106). The resulting supernatant, designated S_1, was then carefully aspirated into a plastic tube and kept on ice until use. The remaining pellet was resuspended in 5 ml of homogenization buffer and then rehomogenized and centrifuged

in the same manner described above. This procedure was repeated 3 further times to obtain supernatant samples designated S_2, S_3, and S_4 respectively.

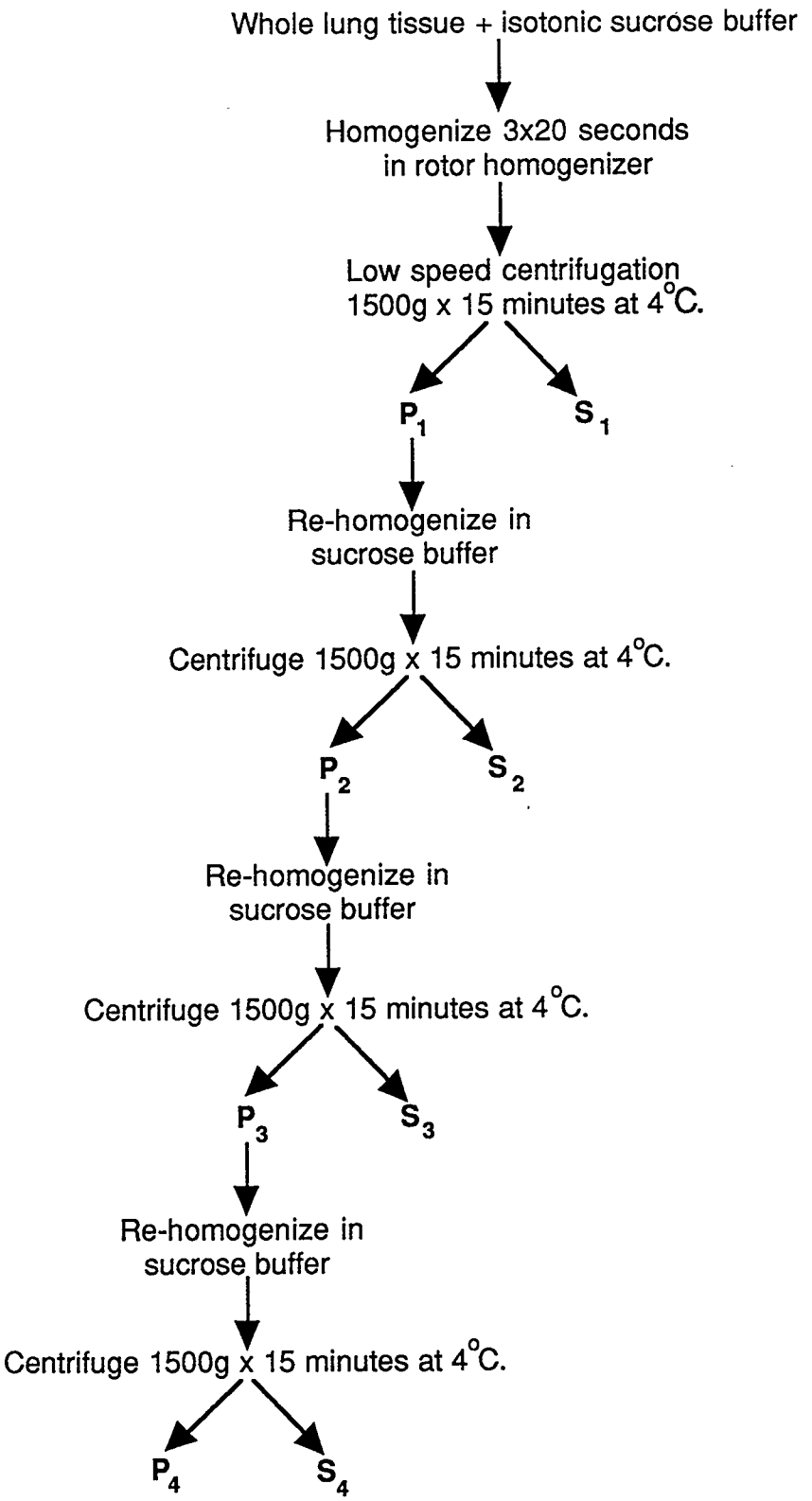


Figure 3.1 Lung supernatant preparation

3.1.2.2 Protein and DNA quantification

Protein and DNA were assayed in the supernatant fractions as described in general methods.

3.1.2.3 Na-K-ATPase Assay

Na-K-ATPase activity was assayed in the supernatant fractions by the technique described in the next section (Na-K-ATPase Assay Implementation and Optimization), using the final optimal assay conditions.

3.1.3 Results

3.1.3.1 Protein and DNA recovery

The protein and DNA recoveries in fractions S_1-S_4 of the lung supernatant preparation are given in table 3.1. The majority of protein and DNA was recovered in the initial fractions, with subsequent re-extractions recovering increasingly less protein and DNA. The combined recovery of the S_1 and S_2 fractions was 93.7% for protein and 84.6% for DNA, assuming that further extractions beyond S_4 would yield minimal further protein or DNA.

3.1.3.2 Na-K-ATPase recovery

The data for recovery of Na-K-ATPase are provided in table 3.2. Na-K-ATPase activity was detected only in fractions S_1 and S_2, there being no net ouabain sensitive ATP hydrolysis in either of fractions S_3 or S_4.

TABLE 3.1
PROTEIN AND DNA RECOVERY FROM FRACTIONS S1-S4 OF LUNG SUPERNATANT PREPARATION

Fraction	Total Protein (mg)	Protein % of total	Total DNA (mg)	DNA % of total
S1	40.87	78.6	1.96	51.3
S2	7.85	15.1	1.27	33.2
S3	2.67	5.1	0.33	8.6
S4	0.62	1.2	0.26	6.8
total	52.01		3.82	

TABLE 3.2
SPECIFIC AND TOTAL Na-K-ATPase ACTIVITIES OF FRACTIONS S1-S4 OF LUNG SUPERNATANT PREPARATION

Fraction	Specific Na-K-ATPase Activity (umole Pi/hr/mg protein)	Total Na-K-ATPase Activity[a] (umole Pi/hr)	Total Na-K-ATPase Activity % of total
S1	1.49	60.9	92.5
S2	0.63	4.9	7.5
S3	ND	-	-
S4	ND	-	-
total		65.8	

a - Total Na-K-ATPase activity was calculated by multiplying the specific activity by the total protein recovery for that fraction.
ND - None Detected

3.1.4 Discussion

The technique described provided a lung supernatant preparation that fulfilled the aforementioned criteria for an adequate lung sample. The sample was a homogeneous liquid and provided for virtually complete recovery of Na-K-ATPase. Volume measurement was accurate with this sample as there was no solid matter present. This is in contrast to several other studies, in which Na-K-ATPase activity was evaluated in gross lung homogenates containing solid matter (107-109). Experience in this study would indicate that volume measurement with pipettes in these situations is difficult and inaccurate. Complete Na-K-ATPase recovery was achieved with only two homogenizations, assuming further extractions beyond the 4 performed would not recover significant further enzyme. Although enzyme recovery is ideally determined based on the amount in the initial gross tissue homogenate, this was not possible here due to the presence of substantial connective tissue debris, making volume measurement inaccurate. Multiple homogenizations of the tissue pellet were therefore performed as an alternative in order to estimate the maximum **extractable** protein, DNA, and Na-K-ATPase activity.

As Na-K-ATPase is a membrane bound enzyme, the object of extraction methods is to obtain a fraction containing plasma membranes. Rotor homogenization has been demonstrated to efficiently rupture plasma membranes of many

tissues (110), and low speed centrifugation, as used here, has been demonstrated to pellet primarily connective tissue, nuclei, and unbroken cells, leaving plasma membranes in the supernatant (105,106). The homogenization buffer used here was designed to be isotonic and of physiologic pH, so as to avoid activation of lysosomal enzymes. The buffer contained DTT, to prevent sulfhydryl group oxidation, EGTA, to bind calcium, which is inhibitory to Na-K-ATPase (86), and protease inhibitors, which, in combination with the cold temperature, limit proteolysis. Further purification steps towards obtaining relatively pure plasma membranes tend to result in significantly reduced overall enzyme recovery, often <5% of that in the initial homogenate (106,111,112). Such subcellular preparations with low enzyme recovery may not represent the remaining majority of unrecovered enzyme and can potentially result in misleading data (111,112). The preparation used here provided for virtually complete recovery of lung Na-K-ATPase at the sacrifice of relative impurity.

Thus, the described technique provided a suitable sample of lung tissue for further analysis. As Na-K-ATPase activity was detected only in the S_1 and S_2 fractions, for all further studies, these two fractions were combined to yield a composite supernatant preparation.

3.2 Na-K-ATPase ASSAY IMPLEMENTATION AND OPTIMIZATION

3.2.1 Introduction

With a method for obtaining a lung sample suitable for further biochemical analysis in place, the next task was to implement and optimize a technique for the assay of Na-K-ATPase activity. There are several methods available for this purpose, all of which rely on the use of ouabain, a specific inhibitor of Na-K-ATPase (113), to differentiate Na-K-ATPase from the many other cellular ATPase's. Of these procedures, however, only those that quantitate ouabain sensitive ATP hydrolysis are suitable for use in noncellular membrane preparations, such as the lung supernatant preparation used here, as the other techniques employ intact cells in their methodology. The generalized ATPase reaction can be summarized as:

$$ATP \xrightarrow[\text{Na-K-ATPase + many others}]{} ADP + P_i$$

where P_i represents free inorganic phosphate. One approach is to monitor spectrophotometrically ouabain sensitive ADP accumulation by linkage to NADH oxidation (114). This technique is limited, however, by variable inhibitory effects of ADP on the ATPase reaction and by sensitivity to NADH oxidase and adenylate kinase contamination from the tissue samples (115,116). A second approach is to quantitate ouabain-sensitive P_i release, either colorimetrically (117) or by using ^{32}P labeled ATP as a

substrate and monitoring $^{32}P_i$ release radiometrically (118). The latter technique was used in these studies as it is reproducible, highly sensitive, produces a stable endproduct, and is of high efficiency for the processing of multiple samples (118-120). Thus, an initial objective of this section was to implement a radiometric P_i release assay for Na-K-ATPase for use with lung supernatant preparation that reproducibly yields linear ouabain-sensitive ATP hydrolysis.

Several investigators, using relatively purified preparations of Na-K-ATPase, have found it necessary to permeabilize samples prior to assay of Na-K-ATPase in order to maximize enzyme activity (121). Plasma membranes, due to their amphipathic nature, tend to form sealed vesicles when in relatively pure form, which can limit the access of hydrophilic ions and chemicals, such as ouabain, to both sides of the membrane (121). Permeablization prior to assay is designed to overcome this problem, and various techniques have been devised to accomplish this, including treatment with detergents, pore-forming ionophores, and sonication (121-125). Each of these methods has potential difficulties. For example, sonication can directly damage enzymes via heating as well as excessive vibrational energy (125) and detergents can inactivate enzymes at higher concentrations (126). A second objective of this section was, therefore, to evaluate several commonly used pre-

treatment steps as to which would yield optimal Na-K-ATPase activity.

Certain characteristics of Na-K-ATPase, such as sodium affinity and ouabain sensitivity, have been found to vary between different preparations of this enzyme. This can in part be accounted for by the fact that different Na-K-ATPase alpha subunit isoforms have distinct affinities for sodium and ouabain (55,60,127) and these alpha isoforms are distributed in a tissue specific manner (63). However, even in a given tissue containing only one type of alpha isoform, different preparations, varying primarily in extent of enzyme purification, can differ in terms of ion and cardiac glycoside sensitivity (127). This may be attributable to differing lipid environments for Na-K-ATPase created in these preparations, which has previously been demonstrated to affect Na-K-ATPase activity (127-131). Although lung contains primarily the alpha-1 isoform of Na-K-ATPase (63), the present lung supernatant preparation has not been examined in terms of ion activation or ouabain inhibition of Na-K-ATPase in order to optimize assay conditions for measurement of Na-K-ATPase activity. Thus, a final objective of this section was to determine the optimal assay conditions for lung supernatant Na-K-ATPase in terms of sodium, potassium, magnesium, and ouabain concentrations.

3.2.2 Methods

3.2.2.1 Na-K-ATPase Assay

Na-K-ATPase activity was assayed using a modified phosphate release assay, using ^{32}P labeled ATP as a tracer, as described (118,119). The final assay conditions were: NaCl 120mM, KCl 20mM, $MgCl_2$ 7.5mM, ATP 4mM with 5-10 uCi gamma (^{32}P)-ATP (ICN Biomedicals) per sample, EGTA 1mM, sodium azide 5mM, imidazole 25mM pH 7.40 ($37^{O}C$), with or without ouabain 2mM. EGTA was included to bind inhibitory free calcium (86) in preference to magnesium (132). Sodium azide was used to inhibit mitochondrial ATPase (133), thus reducing background ATPase activity. The azide concentration used (5mM) results in complete inhibition of mitochondrial ATPase (134).

The procedure was initiated by adding 50 ul (100-150 ug of protein) of lung supernatant sample to 850 ul of assay buffer, with and without ouabain, and incubating for 20 minutes on ice to allow for ouabain binding. The tubes were then transferred to a $37^{O}C$ circulating water bath for 10 minutes to allow for temperature equilibriation. The assay was initiated by adding 0.1 ml of stock ATP solution (40mM with tracer (^{32}P)-ATP), making the final assay volume 1.0 ml, with the above final concentrations of assay components. Aliquots of 0.1 ml were sampled from each tube at one minute intervals for a total of 5 minutes, and placed into previously prepared plastic filtration columns (Quik-Sep

Columns, Isolab) containing 0.5 ml of cold 1.0M perchloric acid, 0.35M NaH_2PO_4. Perchloric acid acts to inactivate the enzyme and NaH_2PO_4 acts to block nonspecific phosphate binding sites on the plastic column. 1.0 ml of a 2% (w/v) activated-charcoal solution was then added, to selectively bind ATP but not P_i (118,135), and the columns were allowed to drip into test tubes. 1.0 ml samples of the eluate were then transferred to scintillation vials for Cerenkov counting (118).

Control tubes, containing all components except the lung sample, were assayed in the presence and absence of ouabain daily in order to correct for nonenzymatic ATP hydrolysis.

3.2.2.2 Analysis of Na-K-ATPase assay data

The analysis of the Na-K-ATPase assay data began by subtracting the appropriate control counts from the sample counts, and then converting the counts to nmole P_i by dividing by the specific activity of concurrently run ATP standards.

The pooled data for a given rat or assay condition was then analyzed using multiple regression analysis with phosphate release as the dependent variable and time as one independent variable (136). A 'dummy' second independent variable (d) was used to account for the influence of ouabain on time dependant phosphate release. This variable takes one of two values: d=1 - no ouabain in assay, d=0 -

ouabain present in assay. The overall regression equation used is:

$y = a + b_1t + b_2(dt)$ where y - P_i release (nmole)
t - time (min)
a - constant
d - dummy variable
d=0 - with ouabain
d=1 - no ouabain
b_1, b_2 - partial regression coefficients

Thus, in the absence of ouabain, d=1, and the regression equation fit to the time-dependant phosphate release data obtained without ouabain becomes:

$$y = a + (b_1 + b_2)t$$

In the presence of ouabain, d=0, which eliminates the last term in the initial regression equation, and makes the equation fit to the time-dependant phosphate release data obtained in the presence of ouabain:

$$y = a + b_1t$$

Thus, the partial regression coefficient b_2 represents the difference in slopes between the rate of phosphate release in the presence and absence of ouabain, which is equivalent to the rate of ouabain-inhibitable ATP hydrolysis, which is taken as the total activity for Na-K-ATPase. This rate was converted to Na-K-ATPase specific activity, expressed in the usual units of umole P_i/hour/mg protein, by transforming b_2 (nmole/min) to umole/hour and then dividing by the amount of protein in the assay. A sample analysis is illustrated in figure 3.2.

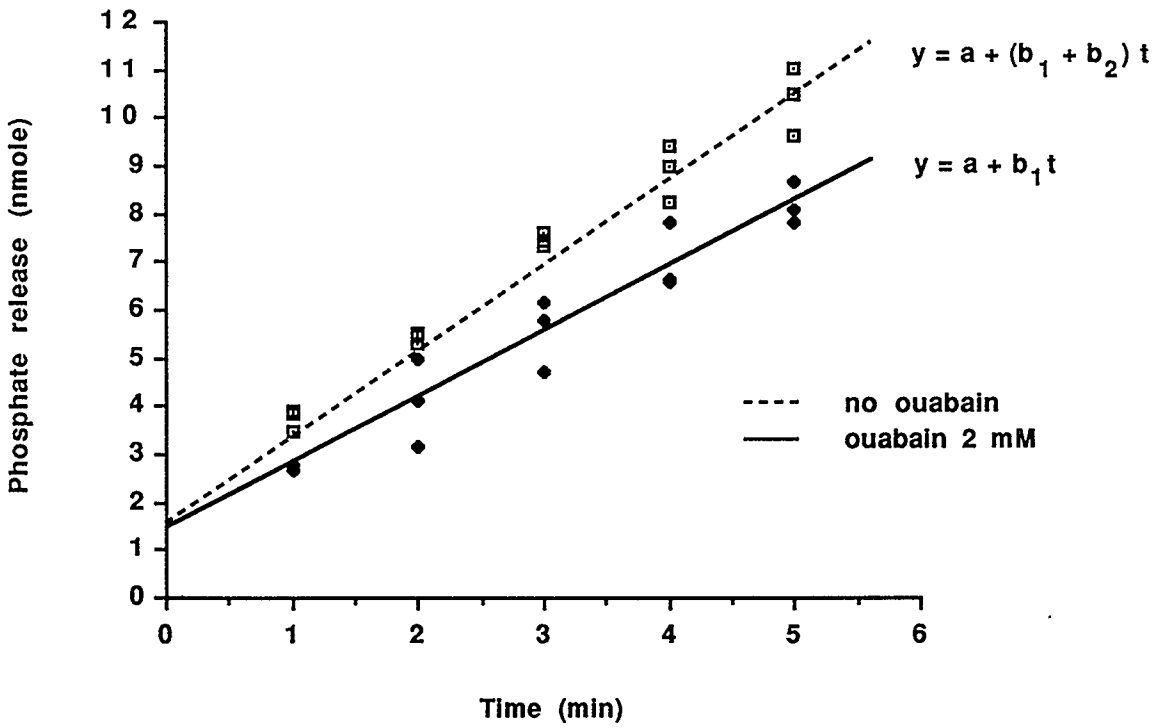


Figure 3.2 Analysis of ouabain-sensitive phosphate release by multiple regression analysis. The release of free phosphate is measured in the presence and absence of ouabain. Multiple regression constrains a common y intercept upon the combined data. The difference in slopes for the regression lines applied to the data obtained in the presence and absence of ouabain represents the rate of phosphate release attributable to Na-K-ATPase.

3.2.2.3 Relative ATPase activities in lung supernatant preparation

To determine the proportion of ouabain, azide, and calcium sensitive ATPase activities in lung supernatant preparation, the ATPase assay was carried out in the presence and absence of these inhibitors. Lung supernatant sample, derived from normal rats, was assayed in triplicate with and without sodium azide (5mM), ouabain (2mM), and $CaCl_2$ (1.0mM) in the assay. The difference in ATPase activity obtained in the presence and absence of each of these substances was taken as ATPase activity sensitive to the given condition. Total ATPase activity was calculated as ATPase activity in the absence of azide, ouabain or calcium, plus the calcium stimulated ATPase activity, as Na-K-ATPase and calcium stimulated ATPase cannot be assayed simultaneously due to the inhibitory effect of calcium on Na-K-ATPase (86).

3.2.2.4 Pre-treatment of lung supernatant preparation

In this series of experiments, lung supernatant preparation derived from normal rats was treated with either detergent, sonication, or the ionophore alamethacin prior to assay for Na-K-ATPase in order to determine the optimal permeablization step. The lung supernatant sample was adjusted to a protein concentration of 3 mg/ml with homogenization buffer prior to all treatments. Control

preparations received no pre-treatment prior to Na-K-ATPase assay.

For detergent pre-treatment, the lung supernatant sample was mixed with a 0.2% (w/v) solution of deoxycholic acid (DOC) (Sigma), prepared in EGTA 1mM, Imidazole 25 mM pH 7.40, to final DOC concentrations ranging from 0-0.075% (w/v). After incubation for 20 minutes on ice with intermittent mixing, 50 ul aliquots were taken for Na-K-ATPase assay as described above. Assays were done in duplicate, with the exception of 0.002%, where only one assay was performed.

For sonication pre-treatment, 0.5 ml of lung supernatant preparation was sonicated (Braun-Sonic 1510) at 75 Watts on ice in 10 second bursts for a total of either 20 or 40 seconds. 50 ul samples were then taken for duplicate assays of Na-K-ATPase activity.

For ionophore pre-treatment, alamethacin (Sigma), diluted in 25% ethanol to a concentration of 20 mg/ml, was added to the lung supernatant sample in final concentrations of 11 or 200 ug/ml (122-124). Ethanol concentration in the assay did not exceed 0.1%. After 20 minutes of incubation on ice, 50 ul aliquots were taken for triplicate assays of Na-K-ATPase activity.

3.2.2.5 Assay optimization

In order to determine the optimal assay concentrations of sodium, potassium, magnesium, and ouabain, lung

supernatant preparation, derived from normal rats, was assayed for Na-K-ATPase activity in the presence of varying concentrations of these substances.

The assay concentration of sodium was varied from 0-148mM (including the 8mM contribution from Na_2ATP), of potassium from 0-30mM, and of magnesium from 0-10mM, while keeping the non-varying ion concentrations constant (sodium 128mM, potassium 20mM, magnesium 5mM) for a given experiment. Ouabain concentration was varied from 0.1-4mM, in order to ensure maximal inhibition of Na-K-ATPase. The time of incubation prior to assay was also varied from 0-40 minutes to ensure adequate time for ouabain binding. 2-4 assays were performed for each condition at each concentration.

The $K_{0.5}$, the concentration of substrate producing half-maximal enzyme activity, was determined for sodium and ouabain by fitting hyperbolic curves to the unaltered concentration-activity data (Sigma-Plot), and deriving from these equations the substrate concentration at half maximal activity.

3.2.2.6 Statistics

The effect of various pre-treatment steps on Na-K-ATPase activity was analyzed by one way analysis of variance (ANOVA) using the Fischer protected least significant difference (PLSD) test to detect differences from the untreated control group (137).

3.2.3 Results

3.2.3.1 General characteristics of Na-K-ATPase assay

The radiometric assay described above reproducibly resulted in linear ATP hydrolysis for up to 15 minutes of incubation and reliably detected an inhibitory effect of ouabain, thus detecting activity attributable to Na-K-ATPase. This was despite the fact that Na-K-ATPase comprised only 9.0% of the total ATPase activity in lung supernatant preparation (figure 3.3). In contrast, azide-sensitive mitochondrial ATPase and calcium stimulated ATPase comprised 53.4% and 3.5% of total ATPase activity respectively (figure 3.3). With the use of azide and calcium chelators in the standard assay buffer, the Na-K-ATPase would presumably comprise a greater proportion of the overall ATPase activity.

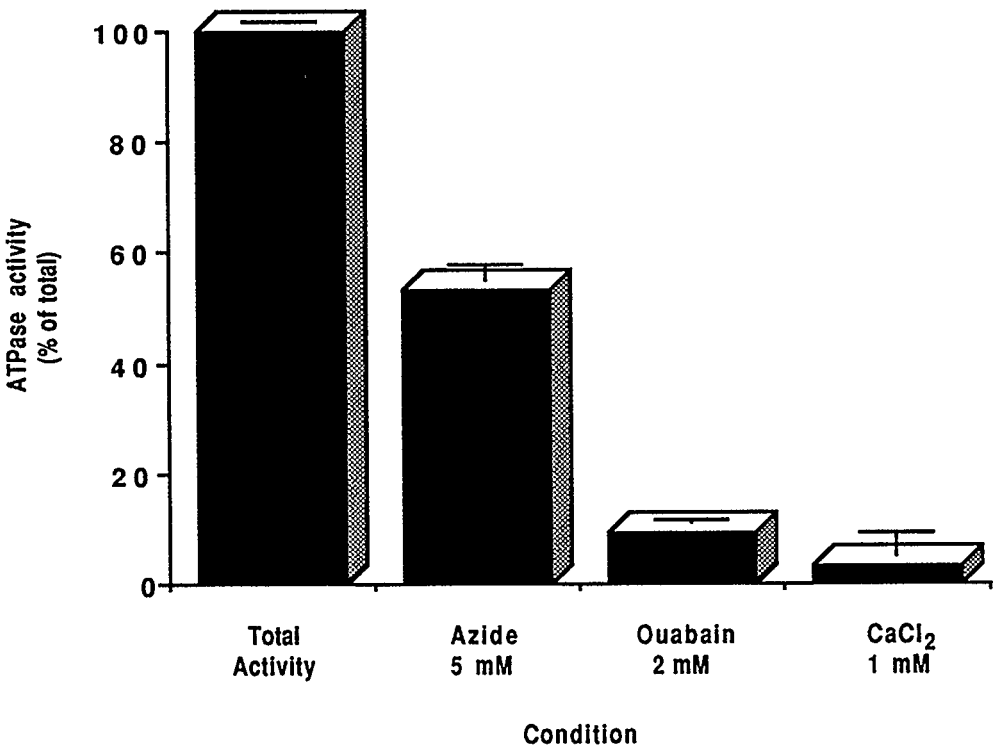


Figure 3.3 Relative ATPase activities in lung supernatant preparation. Lung supernatant was assayed for ATPase activity in the presence and absence of the indicated substances. The difference between the two represents ATPase activity attributable to that condition. Total ATPase activity was 23.7 umole Pi/hr/mg protein.

3.2.3.2 Pre-treatment of lung supernatant preparation

In order to determine the optimal permeabilization step prior to assay for Na-K-ATPase, lung supernatant preparation was pre-treated with several commonly used permeabilization procedures. Pre-treatment of lung sample with the ionic detergent deoxycholic acid for 20 minutes prior to assay resulted in a dose-dependent inhibitory effect on Na-K-ATPase activity up to a concentration of 0.075% (figure 3.4). At no concentration of detergent was there a significantly higher Na-K-ATPase activity relative to the untreated control preparation. Pre-treatment of lung supernatant with sonication, for either 20 or 40 seconds, or with the pore-forming ionophore alamethacin, at concentrations of 11 or 200 ug/ml, also failed to increase Na-K-ATPase activity compared to controls (figure 3.5). 40 seconds of sonication had a significant inhibitory effect on subsequently measured Na-K-ATPase activity. Thus, neither treatment with detergent, sonication, or ionophore proved to significantly increase the subsequently measured Na-K-ATPase activity in lung supernatant preparation.

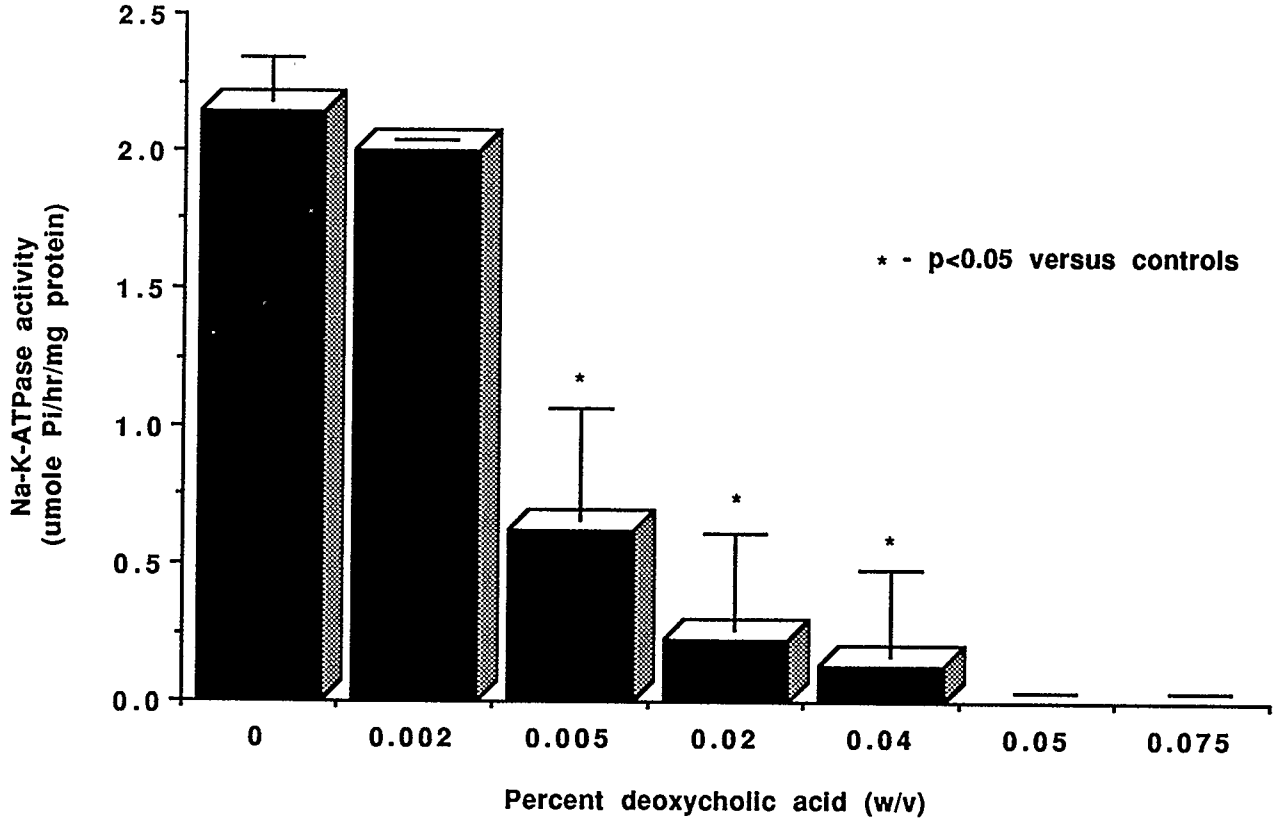


Figure 3.4 Effects of deoxycholic acid pre-treatment on Na-K-ATPase activity in lung supernatant preparation. Lung supernatant preparation was incubated with the indicated concentrations of deoxycholic acid for 20 minutes prior to assay for Na-K-ATPase activity as per *methods.*

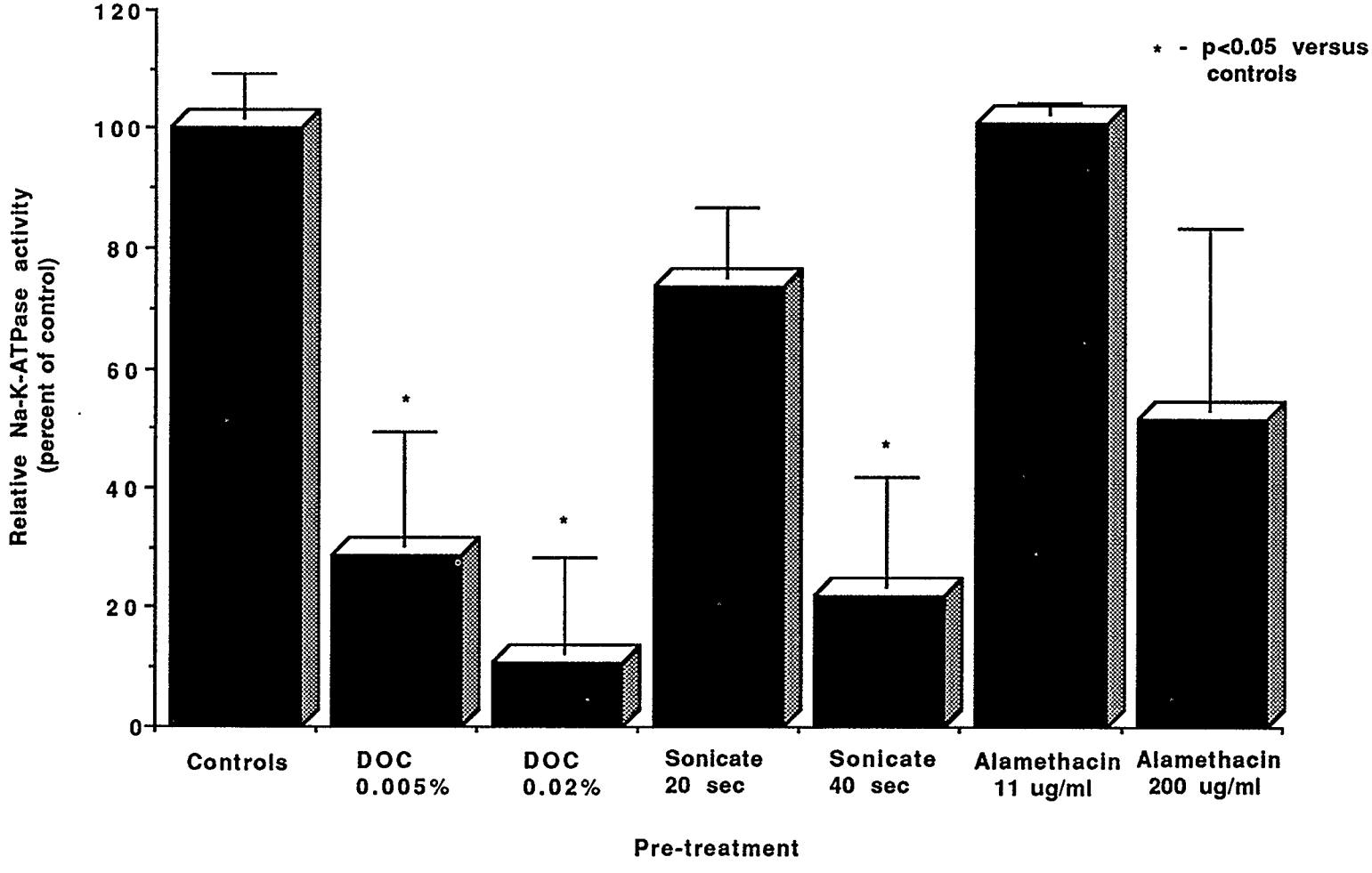


Figure 3.5 Effects of various pre-treatment steps on Na-K-ATPase activity in lung supernatant preparation. Lung supernatant was pre-treated with the detergent deoxycholic acid (DOC) (0.005, 0.02%), sonication (20, 40 seconds), or the ionophore alamethacin (11 or 200 ug/ml) prior to assay for Na-K-ATPase activity as per *methods.*

3.2.3.3 Na-K-ATPase assay optimization

To investigate the sodium, potassium, and magnesium dependence of lung supernatant Na-K-ATPase, the concentrations of these ions were individually varied while concentrations of the other components were kept constant. As expected, Na-K-ATPase activity was dependent on sodium concentration, with maximal activity occurring at a total sodium concentration of 128mM, with a fixed potassium concentration of 20mM (figure 3.6). The $K_{0.5}$ for sodium was estimated to be 50mM. Na-K-ATPase activity was also dependent on the presence of potassium in the assay, however, varied little over potassium concentrations of 5-30mM (figure 3.7). As the Na-K-ATPase activity likely reached a plateau at a potassium concentration <5mM, a $K_{0.5}$ was not calculated for this ion due to the lack of data at lower potassium concentrations. Finally, lung supernatant Na-K-ATPase activity was dependent on the presence of magnesium in the assay, with maximal activity at a concentration of 7.5mM (figure 3.8).

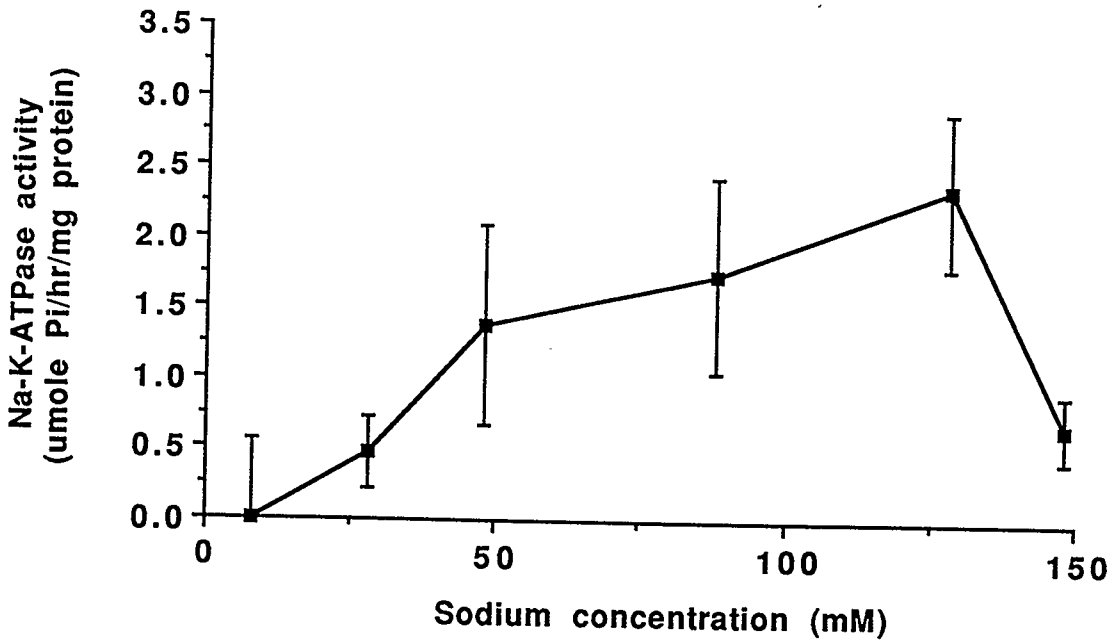


Figure 3.6 Relationship between Na-K-ATPase activity and assay sodium concentration. Lung supernatant was assayed in the presence of varying concentrations of sodium. The concentrations of the other components of the assay were kept constant (K^+=20mM, $MgCl_2$=5.0mM). The $K_{0.5}$ for sodium activation of lung Na-K-ATPase was estimated to be 50 mM.

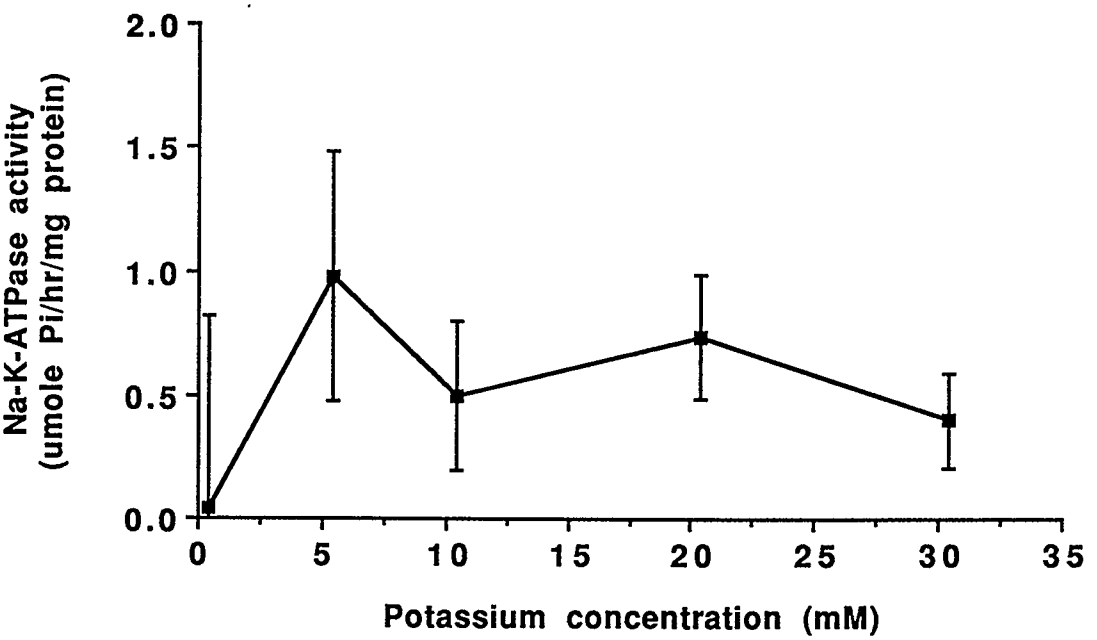


Figure 3.7 Relationship between Na-K-ATPase activity and assay potassium concentration. Lung supernatant was assayed in the presence of varying potassium concentrations. The concentrations of the other components of the assay were kept constant (Na^+=120mM, $MgCl_2$=5.0mM).

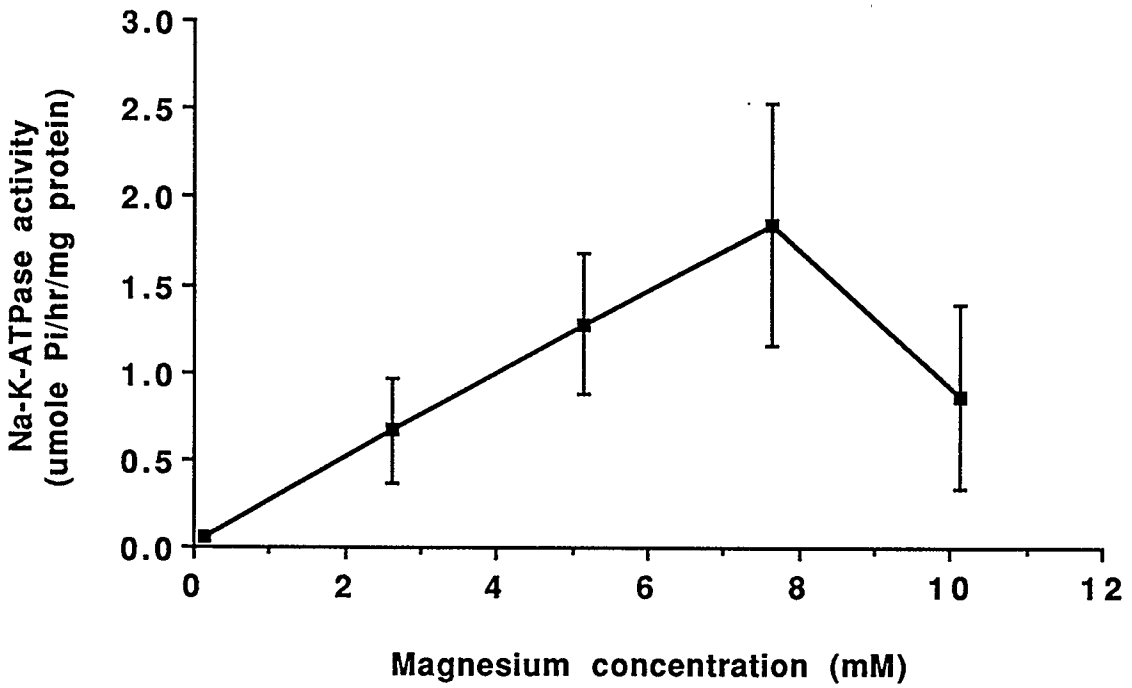


Figure 3.8 Relationship between Na-K-ATPase activity and assay magnesium concentration. Lung supernatant was assayed for Na-K-ATPase activity in the presence of the indicated concentrations of magnesium. The concentrations of the other components of the assay were kept constant (Na^+=120mM, K^+=20mM).

The relationship between ouabain concentration and Na-K-ATPase activity is given in figure 3.9. Maximal inhibition of Na-K-ATPase by ouabain occurred at a concentration of 2mM, with no significant differences in activity between 0.5-4mM. The $K_{0.5}$ for ouabain inhibition was estimated to be 0.15mM. No significant differences in Na-K-ATPase activity were found by varying the total time of incubation with ouabain prior to assay from 10 to 50 minutes (data not shown).

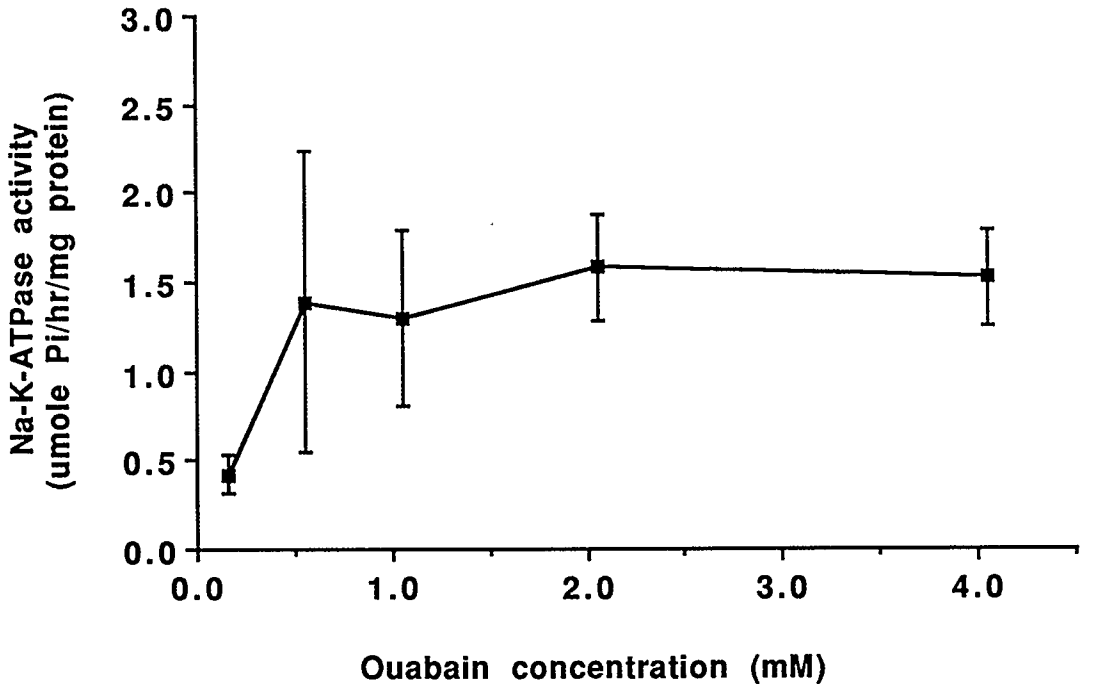


Figure 3.9 Relationship between Na-K-ATPase activity and assay ouabain concentration. Lung supernatant was assayed for Na-K-ATPase activity in the presence of varying concentrations of ouabain. Samples were incubated for a total of 30 minutes prior to assay. The $K_{0.5}$ for ouabain inhibition of lung Na-K-ATPase was estimated to be 0.15 mM.

3.2.4 Discussion

The isotopic phosphate release assay utilized in this study reproducibly yielded linear ATP hydrolysis within the time span of the assay. As well, this technique reliably detected ouabain-sensitive ATPase activity. This in spite of the fact that ouabain-inhibitable ATPase activity comprised a relatively low proportion of total ATPase activity in the lung supernatant preparation (figure 3.3). Both the sodium azide and EGTA included in the assay system act primarily to decrease the activity of such interfering ATPases. Although further purification of the lung supernatant would likely result in a purer preparation of Na-K-ATPase, the sacrifice would be a decreased overall enzyme recovery, with the inherent risk that the eventual preparation may not be representative of the unrecovered enzyme (106,111,112).

The techniques of multiple regression are powerful methods for the combined analysis of multiple sets of data containing multiple independent variables (136). These techniques were used in this study to determine the rate of ouabain-sensitive phosphate release from the combined data of multiple assays, containing multiple time points, for a given rat and assay condition. An advantage of this technique over repeated simple linear regression analysis is that a common y intercept is constrained upon the combined phosphate release data obtained with and without ouabain.

Theoretically, at the moment ATP is added to the samples with and without ouabain, both should start with the same amount of free phosphate (initial P_i is likely derived primarily from non-enzymatic hydrolysis of the stock ATP solution over time). Early trials analyzed by repeated linear regression analysis exhibited random variation in the y intercept in concurrent assays with and without ouabain, which, in light of the increased variability, hampered reliable detection of Na-K-ATPase activity. By utilizing the described multiple regression procedure, the random variation in y intercept position is controlled for, increasing the sensitivity for detection of activity due to Na-K-ATPase.

Several investigators (121-125) have found it necessary to permeabilize membrane preparations, via various procedures, in order to maximize Na-K-ATPase activity. In contrast to these studies, however, neither pre-treatment with the commonly used detergent deoxycholic acid, sonication, nor the pore-forming ionophore alamethacin had a significant beneficial effect on subsequent Na-K-ATPase activity measured in lung supernatant preparation in this study (figure 3.4, 3.5). Further, there were significant inhibitory effects of pre-treatment with deoxycholic acid, at concentrations greater than 0.002%, and sonication (40 seconds). The concentrations of deoxycholic acid (126,138) and alamethacin (122-124), and the time and duration of

sonication (94,125,139) are in the range of those used previously. One factor that may account for this discrepancy is that in most studies in which Na-K-ATPase was found to be in latent form, and thus sensitive to permeabilization procedures, the membrane preparations were relatively purified in nature (121,123,124,126,138), as opposed to the comparatively crude preparation used in this study. Visualized under electron microscopy, such purified membrane preparations have been demonstrated to be largely vesicular in nature, and thus, restrict the access of hydrophilic substrates to one side of the membrane (140). Although crude membrane preparations, such as were used in this study, have not been visualized under electron microscopy, Jones et al (123), found that while alamethacin treatment of a relatively purified preparation of canine sarcolemmal vesicles significantly increased Na-K-ATPase activity, this had little potentiating effect on the activity of crude homogenates, suggesting Na-K-ATPase in these crude homogenates may not be in latent form. Lack of deoxycholic acid potentiation of Na-K-ATPase activity has similarly been observed with crude homogenates of a rat liver cell line (141). Another factor that may account for the discordant effects of permeabilization procedures on crude versus purified membranes is that the homogenization process itself may have significant permeabilization effects. Rotor homogenization, as used here, combines shear

forces with sonication energy to achieve cellular disruption (125). Perhaps the sonication energy was sufficient to achieve adequate membrane permeabilization.

Although the above 3 membrane pre-treatment methods are used with the intention of permeabilizing vesicular membrane structures to assay components (121), each of these techniques has the potential to significantly inhibit enzyme activity, as was observed in this study with increasing concentrations of deoxycholic acid and with sonication (40 seconds). Inhibitory effects of deoxycholic acid (126,138), as well as several other ionic (eg. SDS) and nonionic (eg. Lubrol-PX) detergents on Na-K-ATPase activity have been previously noted (121,138,140). Sonication has been observed to damage protein by altering quaternary structure (142), as well as by oxidizing thiol groups (143). Finally, alamethacin has been demonstrated to significantly inhibit Na-K-ATPase activity in renal membranes, albeit at higher concentrations than used here (144). Thus, each of the permeabilization techniques examined in this study has the potential for inhibitory effects of Na-K-ATPase. As none of these techniques proved to be beneficial for use with lung supernatant preparation, no specific pre-treatment was utilized for all further studies.

The activity of the Na-K-ATPase depends on the presence of sodium, potassium, magnesium, and ATP (129). The optimization steps carried out in this study, in terms of

the assay concentrations of these ions, conform to these requirements. As expected, lung supernatant Na-K-ATPase was sodium and potassium dependent (figures 3.6 and 3.7), with optimal activity occurring at similar substrate concentrations as previously reported (145). The $K_{0.5}$ for sodium was estimated at 50mM, which is in close agreement with a previously reported value of 46mM obtained with rat kidney (127). Magnesium was required in the assay (figure 3.8), with optimal Na-K-ATPase activity occurring at magnesium concentrations greater than those of ATP, indicating, as previously reported (145), a requirement for free magnesium for optimal enzyme activity. The concentration of ATP provided in the assay was such that the reaction velocity was independent of the amount of this substrate (146). Thus, with the excessive substrates provided in the assay system, phosphate release was linear within the time span of the assay, provided that <10% of the ATP was hydrolyzed, in order to avoid the accumulation of inhibitory ADP (145). Ouabain inhibition was maximal at 2mM, with little difference between 0.5-4mM (figure 3.9). The $K_{0.5}$ for ouabain was estimated at 0.15mM, which is similar to that reported for other alpha-1 isoform-containing tissues (55,147,148). With the above information, optimal conditions for the assay of Na-K-ATPase activity can be applied for use with the particular species, tissue source, and preparative method used in these studies.

Together, these studies demonstrate that the radiometric phosphate release assay provides a reliable and reproducible means of determining the specific activity of Na-K-ATPase in lung supernatant preparation.

CHAPTER 4

Na-K-ATPase ACTIVITY DURING THE RESOLUTION OF HIGH PERMEABILITY PULMONARY EDEMA

4.1 INTRODUCTION

As discussed in chapter 1, numerous studies have demonstrated the importance of active sodium transport in the process of lung liquid clearance. The Na-K-ATPase is an important part of this active transport process, being the only identified mechanism for the clearance of sodium into the interstitium once absorbed from the apical surface of the alveolar membrane. Virtually all studies examining sodium transport in intact lung preparations, however, have utilized artificially induced alveolar flooding to re-create the situation presumed present in pathologic states of pulmonary edema. While this may simulate liquid clearance in normal, undamaged lungs, this is likely quite unlike situations involving lung injury where there may be epithelial damage or dysfunction. The study of Matthay *et al* (5) would suggest that, even in the presence of lung injury, lung liquid, and thus sodium transport can occur, and may be necessary for the successful recovery from pulmonary edema. Further, the study of Nici *et al* (94) provides evidence for a potential regulatory role for Na-K-ATPase during the recovery process after lung injury. As active sodium transport has not been adequately studied in the setting of pathologic states of pulmonary edema, the primary objective of this section was to evaluate the

activity of lung Na-K-ATPase, used as an index of active sodium transport, during the recovery from high-permeability pulmonary edema. The agent chosen to induce such high-permeability edema, thiourea, has been well characterized as a specific toxin to the microvascular endothelial barrier (149-151) which results in an increased permeability pulmonary edema (149,152,153) whilst producing no changes in microvascular hydrostatic pressure (152,154).

4.2 METHODS

4.2.1 Protocol

In order to determine the influence of pulmonary edema on active sodium transport in the lung, the activity of Na-K-ATPase was examined in lung supernatant preparations derived from rats treated with either thiourea or normal saline. High-permeability pulmonary edema was induced in rats by the intraperitoneal injection of thiourea (1.5 mg/Kg body weight) dissolved in sterile normal saline. Control rats received an equivalent volume of the saline vehicle. Intraperitoneal injection was confirmed in 6 rats (3 thiourea, 3 saline) by adding bromophenol blue (5 ug/ml) to the injectate and visualizing the dye within the peritoneal cavity at sacrifice. At 4, 8, 12, 48 or 96 hours after injection (n=6 rats for each time point in each treatment group), the animals were anesthetized with pentobarbital (60 mg/Kg intraperitoneal) and exsanguinated via transection of the abdominal aorta and inferior vena cava. Any pleural

effusion was carefully aspirated into graduated syringes for volume measurement, and later assayed for protein concentration using the Biuret procedure (155). The lungs and mediastinal structures were then removed in toto and the right and left lungs separated via transection close to the hilum. The larger right lung was used for measurement of a wet/dry lung weight ratio (see chapter 2), and the smaller left lung was used for the preparation of a lung supernatant sample (see chapter 3). The lung supernatant preparation was then assayed for Na-K-ATPase activity (see chapter 3) and protein and DNA concentrations (see chapter 2). Na-K-ATPase activity was normalized to DNA because the protein concentrations of the lung supernatant samples of thiourea treated animals were significantly higher compared those of the saline treated animals due to the induction of increased lung protein permeability. To further examine the early changes in edema induction, additional rats were injected with thiourea or saline and examined for wet/dry ratio at 1 and 2 hours (n=3 at each time point in each group).

4.2.2 Lung histology

To determine the distribution of thiourea-induced lung edema, the lungs of rats injected with thiourea were examined at 2 (n=2) and 24 (n=2) hours after injection. Two control rats, injected with normal saline, were also studied at 2 hours. The lung fixation process followed the freeze-substitution technique described by Michel et al (156). In

brief, under pentobarbital anesthesia, the rats were first exsanguinated via transection of the abdominal aorta. A 16-gauge needle cuffed with plastic tubing was then inserted into a tracheostomy, secured with ties, and the lungs were then inflated with air to a constant distending pressure of 20 cmH_2O. The inflated lungs, with attached cannula, were then dissected from the thoracic cavity and immediately immersed in liquid nitrogen. The frozen lungs were then immersed in Carnoy's fixative for 2 days at $-70^{O}C$, followed by 2 days at $-20^{O}C$, and finally for 1 day at $4^{O}C$. The Carnoy's solution was then replaced with 70% ethanol, which was in turn replaced with 10% buffered formalin over 2 days. One saggital block from the right lung and three transverse blocks from the left lung, all approximately 1 mm thick, were then taken, embedded in paraffin, and 5 um sections obtained for routine staining with hematoxylin and eosin.

4.2.3 Statistics

Differences between thiourea and saline (control) groups for wet/dry ratio, pleural fluid studies, DNA recovery, and enzyme activity, were analyzed using two-way analysis of variance (ANOVA), with time and treatment group as independent variables (157). Multiple comparisons were made using the Fischer protected least significant difference (PLSD) test where indicated (137).

4.3 RESULTS

4.3.1 Model of high permeability pulmonary edema

Thiourea, at a dose of 1.5 mg/Kg body weight, reliably resulted in substantial, reversible pulmonary edema. Rats injected with thiourea exhibited decreased activity and increased respiratory effort, these symptoms maximal approximately 4 hours after injection. Saline treated rats appeared normal at all times. There were no deaths in either of the groups. The mean wet/dry lung weight ratios (Q_{wl}/dQl) of the saline treated animals were relatively stable over time (figure 4.1), with a pooled mean value of 3.81 +/- 0.05. In contrast, the wet/dry ratios of the thiourea treated rats increased significantly, peaking at a mean value of 5.98 +/- 0.09, four hours after injection, with gradual recovery over 48 hours. The difference in wet/dry ratios over 96 hours between the thiourea and saline groups was statistically significant ($p<0.0001$ by two-way ANOVA).

Figure 4.2 illustrates the light microscopy of lung tissue obtained after thiourea or saline injection. Lungs from saline-treated animals appeared normal with thin alveolar septae and no interstitial edema (figure 4.2a). In contrast, lungs from thiourea-treated animals demonstrated substantial interstitial edema with prominent bronchovascular cuffing (figure 4.2b). By 24 hours, these abnormalities had largely resolved (figure 4.2c).

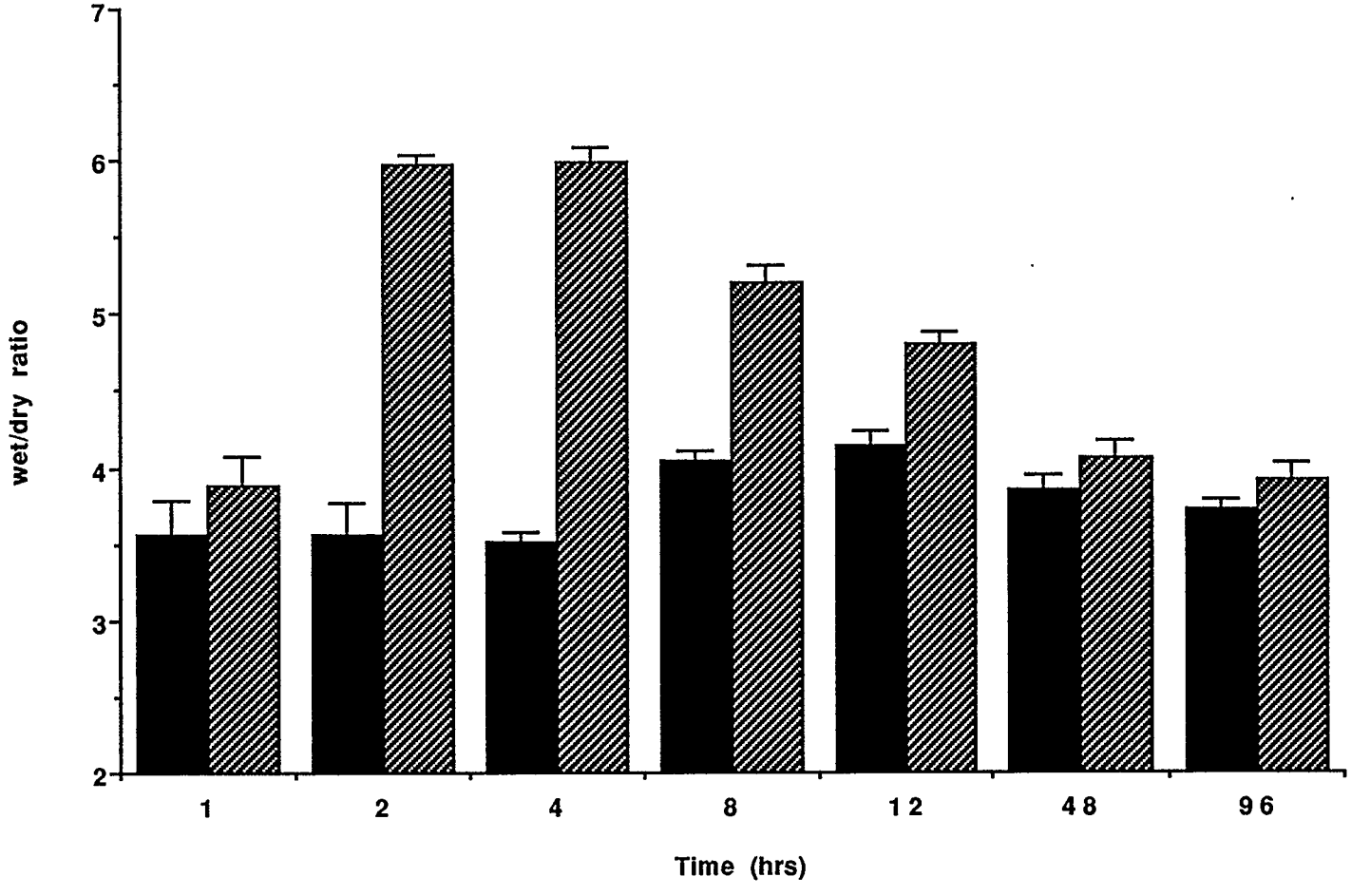
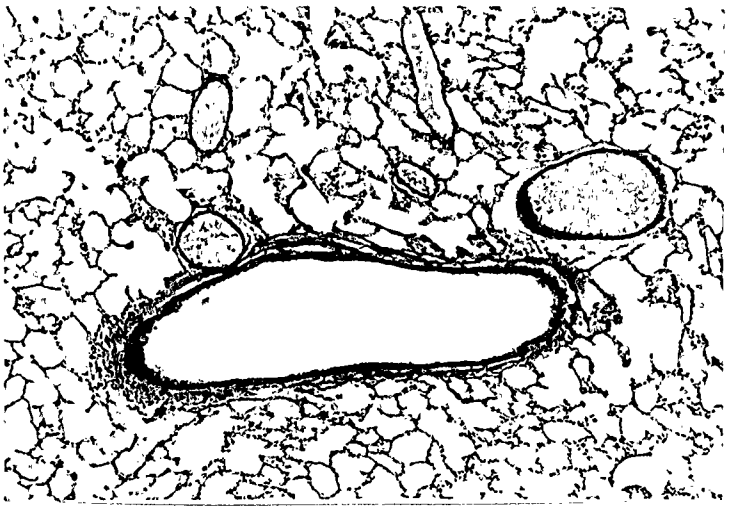


Figure 4.1 Mean wet/dry lung weight ratios (Q_{wl}/dQl) over 96 hours for rats treated with thiourea (▨) and normal saline (■). The thiourea group developed substantial pulmonary edema, peaking at 2-4 hours, with gradual recovery over 48 hours. Wet/dry ratios of control rats were relatively stable over time. The difference in wet/dry ratios over time between the thiourea and saline groups was significant ($p<0.0001$, two-way ANOVA).

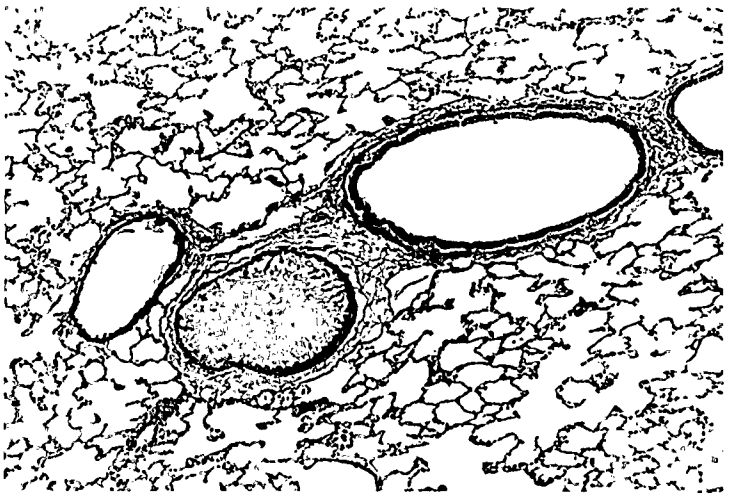
Figure 4.2 Representative light micrographs of lung tissue from rats at different stages of lung injury. Magnification x100. **A:** Saline control (2 hours after treatment) - normal alveolar septae. **B:** Thiourea (2 hours after treatment) - bronchovascular cuffing and thickened alveolar septae suggestive of interstitial edema. **C:** Thiourea (24 hours after treatment) - minor bronchovascular cuffing, normal alveolar septae.

A



B

C



Thiourea treatment also resulted in the accumulation of large pleural effusions. Pleural fluid volume was maximal at the 8-hour time point (5.3 +/- 0.7 ml), with resorption by 48 hours (figure 4.3). Mean pleural fluid protein concentration was maximal at 2 hours (5.2 +/- 0.4 gm/dl), with a significant trend towards gradually decreasing protein concentrations over the next 10 hours ($p<0.02$, one-way ANOVA) (figure 4.4). The effusions were exudative in nature, with the ratio of pleural fluid/serum protein concentrations ranging from 0.79-0.91.

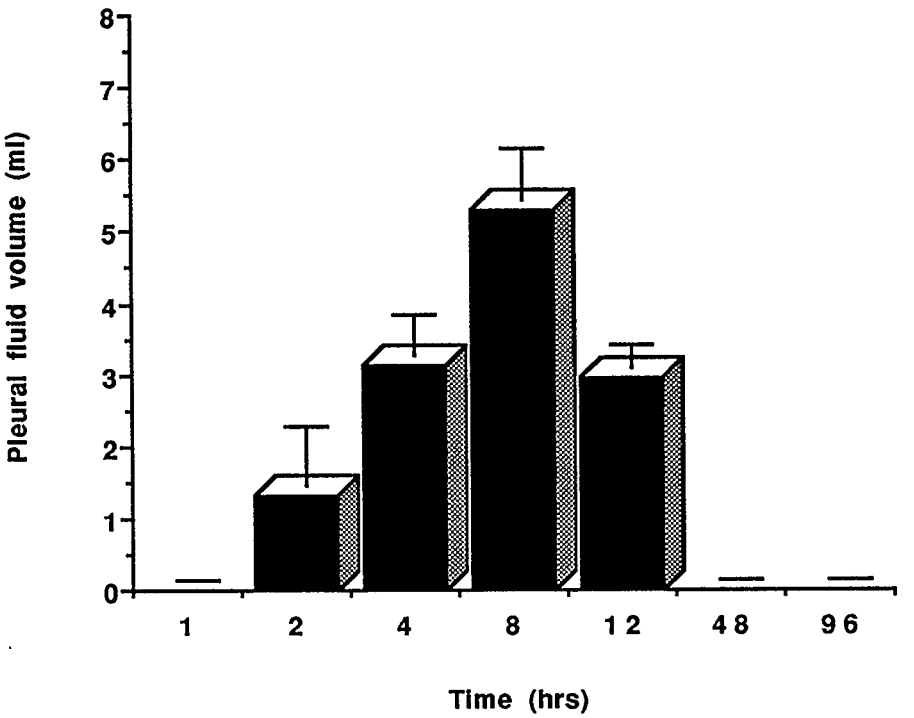


Figure 4.3 Mean pleural fluid volumes over 96 hours for thiourea treated rats. Pleural fluid volume peaks at 8 hours with resorption by 48 hours. Saline treated rats had no detectable pleural fluid at any time point (data not shown).

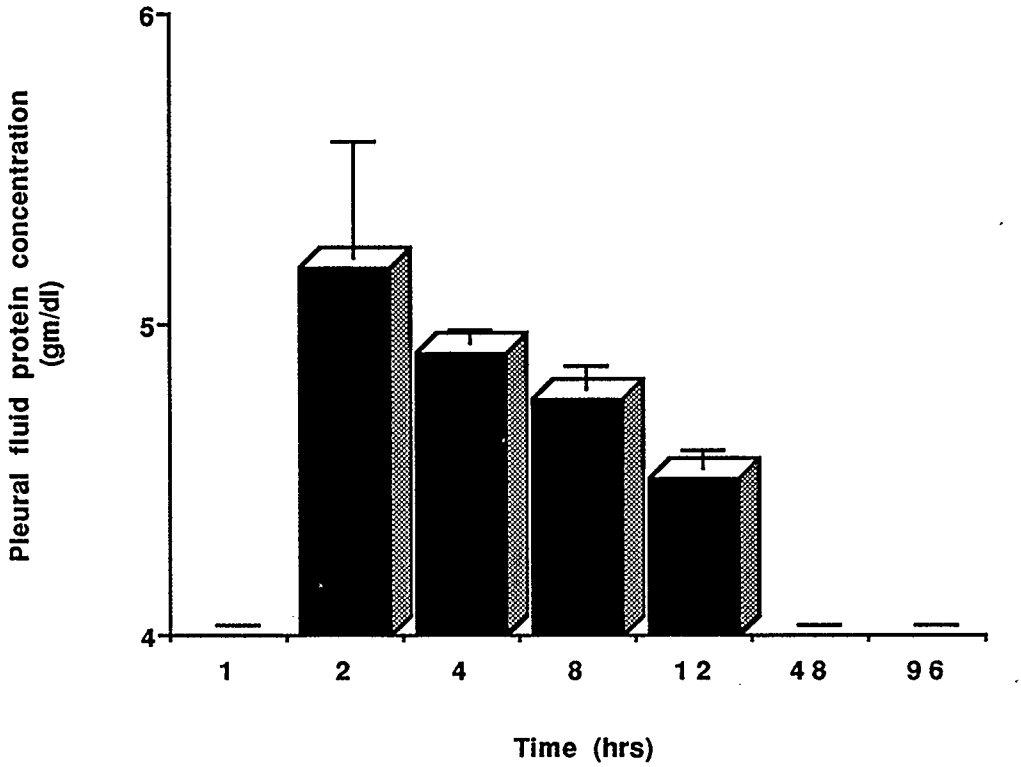


Figure 4.4 Pleural fluid protein concentration over 96 hours for thiourea treated rats. There is a significant trend towards declining protein concentration over 2-12 hours ($p<0.02$, one-way ANOVA). The mean serum protein concentration was 5.70 +/- 0.16 gm/dl.

4.3.2 Na-K-ATPase activity during edema recovery

Figure 4.5 provides the mean Na-K-ATPase activities for the thiourea and saline treatment groups over 96 hours. Enzyme activity was relatively stable in the control group, with a pooled mean value of 17.1 +/- 1.9 umole Pi/hr/mg DNA. In contrast, there were significant increases in Na-K-ATPase activity over time in the thiourea group ($p<0.0001$, thiourea versus saline groups, two-way ANOVA). Enzyme activity peaked at 12 hours in the thiourea group (60.1 +/- 14.7 umole Pi/hr/mg DNA) followed by recovery over 96 hours. The increases in activity were not due to changes in lung DNA content, as DNA recoveries were similar between the thiourea and control groups (figure 4.6), the only exception being at 96 hours, where there was a significantly higher mean DNA recovery in the thiourea group. The relationship between edema recovery and lung Na-K-ATPase activity for the thiourea-treated animals is illustrated in figure 4.7. Na-K-ATPase activity was increasing as pulmonary edema was resolving, as indicated by decreasing wet/dry ratios.

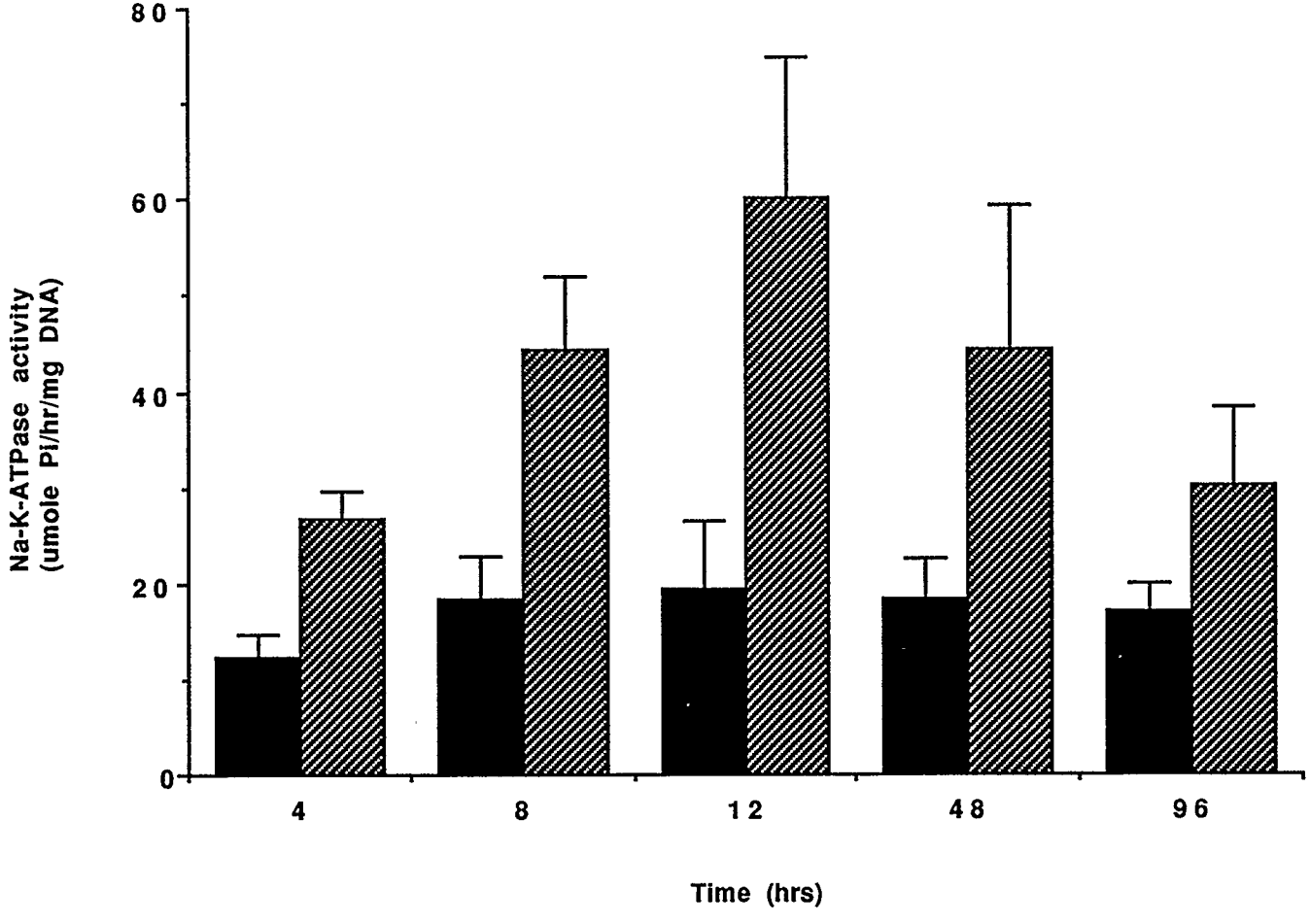


Figure 4.5 Mean Na-K-ATPase activity over 96 hours for rats treated with thiourea (▨) and normal saline (■). There was a significant increase in activity over time in the thiourea group relative to the saline controls ($p<0.0001$, two-way ANOVA), with Na-K-ATPase activity peaking at 12 hours, followed by recovery over 96 hours.

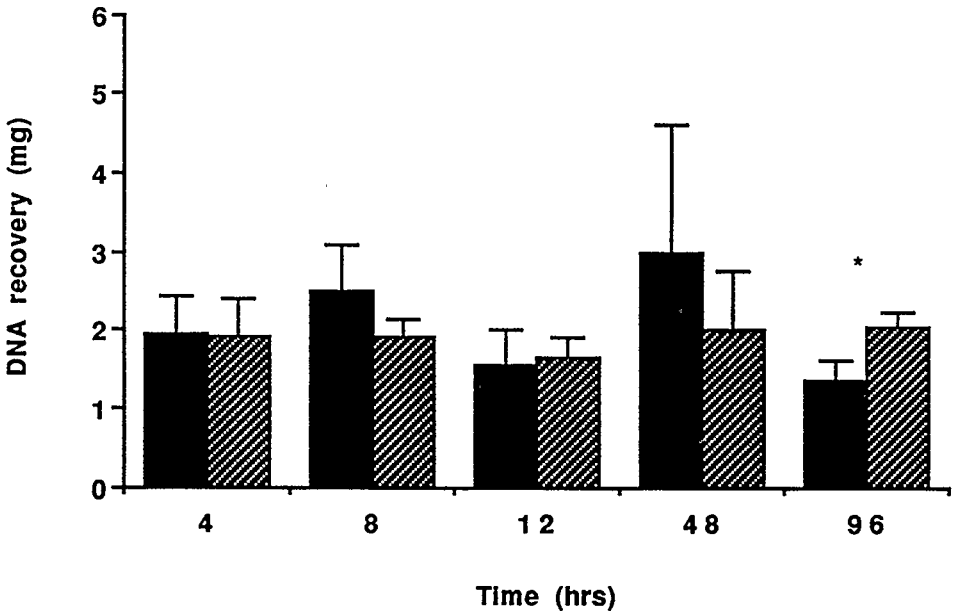


Figure 4.6 Mean DNA recovery for time points 4-96 hours for the thiourea (▨) and saline treated groups (■). Only the 96 hour groups significantly differed from each other (* - $p<0.05$ Fischer PLSD test).

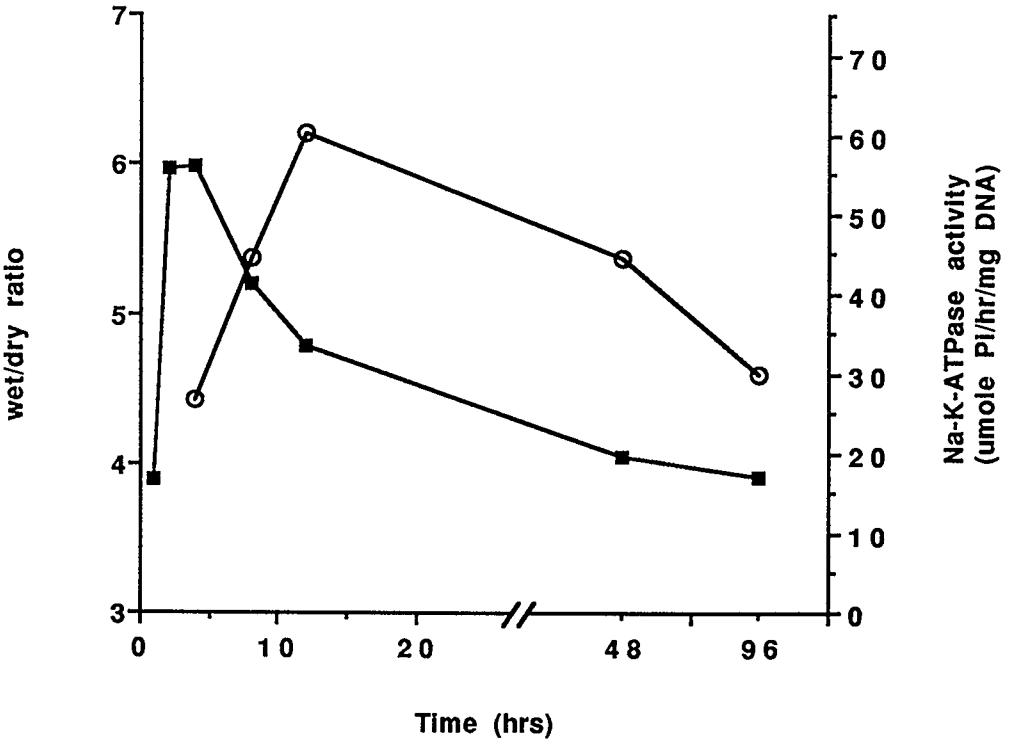


Figure 4.7 Relationship between the time courses for mean wet/dry ratio (■) and mean Na-K-ATPase activity (o) for the thiourea treated animals. Na-K-ATPase activity was increasing when pulmonary edema was resolving, as indicated by decreasing wet/dry ratios.

4.4 DISCUSSION

Numerous studies have demonstrated the importance of active sodium transport in the process of lung liquid clearance using models of alveolar flooding (see chapter 1). The Na-K-ATPase is a key component of this transport process, as it is the active mechanism by which sodium is cleared from alveolar epithelial cells into the interstitium. The activity of this enzyme, thus, provides a useful measure of active sodium transport for use with whole lung studies.

This study demonstrates that Na-K-ATPase activity increases substantially during the resolution of thiourea-induced, high-permeability pulmonary edema. As Na-K-ATPase is a key component of the active sodium transport mechanism in the lung, these results support the hypothesis that active sodium transport plays a role in the recovery from pulmonary edema. The major evidence for this lies in the relationship between the time course of edema recovery, as reflected by decreasing wet/dry lung weight ratios, and the time course of activation of lung Na-K-ATPase (figure 4.7).

The thiourea model of lung injury used in this study is a well-characterized model of pulmonary edema (150,152,158). At the dose used in this study thiourea resulted in substantial and reversible lung edema (figure 4.2). There was no mortality among the thiourea treated animals, thus, eliminating any exclusion bias in the analysis. Thiourea

has been characterized as a relatively specific toxin, selectively altering the ultrastructure of the pulmonary microvascular endothelial barrier, and having virtually no morphological effects on the alveolar epithelial barrier (149-151), although subtle functional abnormalities of these epithelial cells may be induced as well (159). The edemogenic effects of thiourea are purely due to changes in permeability with no changes in pulmonary microvascular hydrostatic pressures (149,152-154). These changes are reversible once the thiourea is metabolized or excreted (151). Although the biochemical mechanism for toxicity of thiourea has not been definitely established (160), possibilities include the oxidation of thiourea to either atomic sulfur (161) or cyanide-containing compounds (162) or the creation of oxygen free radicals (163). Thus, although having no direct clinical counterpart, this model of thiourea induced pulmonary edema shares several aspects with human lung injury (ARDS) - high-permeability pulmonary edema, endothelial cell damage, and the possible involvement of oxygen free radicals. As well, treatment with thiourea increases the rate of airway clearance of ^{99}Tc-DTPA (164), an effect also seen in patients with lung injury (165).

In addition to the pulmonary parenchymal changes induced by thiourea, protein-rich pleural effusions were observed to accumulate in this and other studies (158). Although the mechanism by which these effusions form is

uncertain, the movement of lung edema into the pleural space has been identified as an important clearance mechanism in sheep (166,167). In this study, pleural fluid volumes peaked at a time 4 hours after the peak of lung edema (4 hours), which is consistent with this mechanism. Richter (158) similarly found, using the same model of lung injury, that pleural fluid begins to collects only when pulmonary edema begins to recede.

An interesting finding in this study was the relatively large degree of variability in Na-K-ATPase activities at each time point during the recovery from thiourea-induced edema (figure 4.5). This variability may be due to variation in enzyme activity between rats and/or variation in the estimate for enzyme activity for individual rats. In order to address these two possibilities, a one-way ANOVA of the enzyme activities of the 6 rats examined 12 hours after injection with thiourea was performed. Each of the 3 trials of the Na-K-ATPase assay performed for each rat was analyzed separately, so as to yield 3 estimates of activity for each rat. The majority (74%) of the variability in the overall mean activity for the time point was accounted for by inter-rat variability rather than intra-rat variability in the enzyme assays. Factors that may account for this variability in Na-K-ATPase activity between animals may include variation in the absorption, distribution, metabolism, or pulmonary response to thiourea, which may

result in variable durations and intensities of edemogenic stimuli to Na-K-ATPase activation. Variation in intrinsic lung liquid or sodium clearance capacity could similarly result in variable exposures to edemogenic stimuli.

The results of this study raise several questions as to the mechanisms behind the observed activation of lung Na-K-ATPase in this model of edema. Firstly, does the observed activation of lung Na-K-ATPase simply reflect a direct effect of thiourea on this enzyme, or rather, are the changes in enzyme activity secondary to the physiologic alterations associated with lung injury, in particular, edema? There are no reports of direct effects of thiourea on the Na-K-ATPase. In the absence of a direct effect, several other physiologic alterations could influence the activity of this enzyme. Catecholamines (98,99) and both gluco- and mineralocorticoids (98) are known activators of Na-K-ATPase that are found in increased circulating levels in critically ill patients. Similarly, ADH is capable of inducing increased synthesis of Na-K-ATPase and is released in significant quantities during hypoxemia (96). Although the identification of the precise mediators altering Na-K-ATPase activity during pulmonary edema resolution is beyond the scope of this study, determining whether or not the observed activation of Na-K-ATPase is likely consequent to the presence of edema seems desirable so the results of this

study may be more generalizable to other states of lung edema.

A second question regarding the observed changes in whole lung Na-K-ATPase activity concerns the relative contribution of alveolar inflammatory cells to this activity. Alveolar inflammatory cells, predominantly neutrophils, have been noted to accumulate within the alveoli of patients with lung injury (168) as well as in several animal models of pulmonary edema (11,169). A significant increase in the number of bronchoalveolar lavage (BAL) inflammatory cells has been noted after treatment with approximately equimolar doses of the related thiourea alpha-napthylthiourea (ANTU) (170). An increase in total lung Na-K-ATPase could reflect increases in alveolar inflammatory cell number and/or increases in specific enzyme activity per cell. The contribution of these inflammatory cells to changes in lung Na-K-ATPase activity could be evaluated by BAL, followed by evaluation of total cell number and enzyme activity (171).

The contribution of intravascularly located cells (red and white blood cells, platlets) to total lung Na-K-ATPase activity is expected to be minimal in this edema model, as the animals were exsanguinated prior to lung removal. Further, there was no correlation between the mass of blood in the lungs, calculated from the wet/dry ratio analysis, and Na-K-ATPase activity ($r=0.012$, $p=NS$). A correlation

between these two variables would be expected if blood components contributed significantly to lung Na-K-ATPase activity.

Further cellular localization of Na-K-ATPase activity changes becomes difficult with this in vivo model of pulmonary edema. Although there is a technique for the isolation of AIIC, and these cells are known to possess significant Na-K-ATPase protein (92), there is very limited experience with the isolation of these cells from injured lungs (35). Further, limited AIIC recovery (49) and questions regarding the functional status of AIIC in vitro (see chapter 1) further limit the analysis of this cell type. While the alveolar type I cell comprises the vast majority of the alveolar surface area (47,48), and would be most interesting to analyze with regards to Na-K-ATPase activity, there is, as of yet, no reliable method for their isolation.

A final question that arises from this study relates to the mechanism behind the changes in pulmonary Na-K-ATPase activity during edema recovery. Is the observed increase in lung Na-K-ATPase activity secondary to an increase in enzyme quantity and/or an increase in the specific molecular activity (turnover rate) of the enzyme? The answer to this question is important in terms of further study into the regulatory factors mediating the changes in lung Na-K-ATPase activity. Changes in enzyme quantity are likely to be

induced by factors capable of altering gene expression for this protein, whereas changes in specific molecular activity are more likely to be induced by agents directly interacting with the enzyme (see chapter 1, section 2b - Regulation of Na-K-ATPase activity). Chapman et al (93) recently addressed this same question with regards to the increases in AIIC Na-K-ATPase activity that occur during the transitions from both fetal to neonatal and neonatal to adult life. It was found that perinatally, cellular Na-K-ATPase activity increased secondary to an increase in specific molecular activity (turnover rate), whereas postnatally, cellular activity increased secondary to an increase in enzyme number (93). Thus, there is evidence that both of these mechanisms may potentially be active in the lung.

In summary, this study demonstrates that pulmonary Na-K-ATPase activity increases during the resolution from thiourea-induced, high-permeability lung edema. This supports the hypothesis that active sodium transport is operative during edema resolution. In an effort to more fully understand the mechanisms behind this increase in enzyme activity, the following sections will address: 1) the direct effects of thiourea and the indirect effects of alveolar flooding on Na-K-ATPase activity, 2) the contribution of alveolar inflammatory cells to total lung

Na-K-ATPase activity, and 3) whether or not enzyme quantity changes during edema recovery.

CHAPTER 5

THE DIRECT EFFECT OF THIOUREA AND THE INDIRECT EFFECTS OF ALVEOLAR FLOODING ON LUNG Na-K-ATPase ACTIVITY

5.1 INTRODUCTION

In the previous section, it was demonstrated that lung Na-K-ATPase activity increased during the resolution of high permeability pulmonary edema induced by thiourea. As the Na-K-ATPase is an important part of the mechanism of active sodium transport, the observed activation of this enzyme supports the hypothesis that active sodium transport is operative during edema resolution. In order to be consistent with this hypothesis, however, the primary stimulus for the activation of Na-K-ATPase must be the presence of either edema itself or physiologic alterations associated with edema (eg. hypoxia, neurohormonal changes). Specifically, the presence of thiourea should not be required for the activation of Na-K-ATPase. As radiolabeled thiourea has been demonstrated to bind to unspecified macromolecules in the lung (160,172-174), the possibility exists that a direct interaction between thiourea and lung Na-K-ATPase may be mediating the changes in enzyme activity observed during edema recovery in this study.

Given the above information, an obvious question that arises concerning the results of the previous section is whether the observed activation of lung Na-K-ATPase during edema resolution reflected a direct effect of thiourea on the enzyme, or rather, were the changes in activity

consequent to either the presence of, or the physiologic alterations associated with, edema? In an effort to address these possibilities, the objectives of this section were, firstly, to evaluate the in vitro effects of thiourea on lung Na-K-ATPase, and secondly, to determine whether the presence of alveolar flooding alone, in the absence of lung injury, could activate lung Na-K-ATPase.

5.2 METHODS

5.2.1 in vitro effects of thiourea on Na-K-ATPase activity

In order to determine the effects of thiourea on lung Na-K-ATPase activity in vitro, lung supernatant preparation, derived from normal rats, was assayed for Na-K-ATPase activity in the presence or absence (control) of thiourea. Thiourea was added to the standard assay buffer to final concentrations of 2.5 ug/ml or 25 ug/ml. The lower concentration was based on a distribution of the edemogenic dose of thiourea in the total body water of rats, as has been previously demonstrated (172). The 10-fold higher concentration was used to account for potential concentration of thiourea within lung tissue, as has been suggested from in vivo studies (172,173). Lung supernatant was incubated with assay buffer +/- thiourea on ice for a total of 60 minutes prior to assay of Na-K-ATPase activity (see chapter 2). Lung supernatants derived from 5 normal rats were used for the study, each sample being assayed in duplicate for each of the conditions.

5.2.2 Alveolar flooding model

In order to determine the effects of alveolar flooding on lung Na-K-ATPase, a well-characterized sheep model of alveolar flooding (18) was adapted for use in the rat. Na-K-ATPase activity was determined in lung supernatant preparations derived from rats which either were instilled with a protein-containing solution (n=6) or underwent sham preparation (controls) (n=6).

Rats, anesthetized by spontaneously breathing halothane (4% in air), were intubated via a tracheostomy with a blunt 16-gauge needle cuffed with plastic tubing. The rats were then ventilated (Harvard rodent ventilator model 683) with a 50:50 air-oxygen mixture at a tidal volume of 10 ml/Kg, rate of 95 breaths/minute, and a positive end-expiratory pressure of 3 cmH_2O. The halothane was adjusted to maintain adequate anesthesia and blood pressure (range 0.7-2%). The carotid artery was then cannulated with 26-gauge Teflon tubing (Small Parts Inc.) previously heparinized after coating with TDMK heparin binder (Polyscience Inc.). Lines from the carotid artery and airway were then connected to transducers (Statham) previously zeroed to the midchest level, and mean arterial and airway pressures monitored (Grass polygraph). Rectal temperature was continuously monitored (YSI tele-thermometer) and normothermia was maintained with a heat lamp.

After a baseline period of at least 15 minutes, during which time temperature and mean arterial and peak airway pressures were stable, the lungs were instilled, via the tracheostomy tube, with 5 ml/Kg of a 5% (w/v) solution of bovine serum albumin (BSA) in sterile normal saline. The rats were rotated during instillation to even the distribution of the solution between right and left lungs. Control animals underwent the same preparation but were not instilled. The animals were then ventilated with the same initial oxygen concentration for 45 minutes. Arterial blood gases were drawn from the carotid line at baseline and 25 and 45 minutes after instillation, or after an equivalent time in the controls. Fluid support, provided to all animals, consisted of 2 ml of Ringers lactate solution given via the arterial line at baseline and 25 minutes. After 45 minutes, the abdomen was opened, a 3-4 ml blood sample was drawn into a heparinized syringe, and the animal was then exsanguinated via transection of the abdominal aorta and inferior vena cava. The lungs and mediastinal contents were then carefully dissected from the thoracic cavity, the right and left lungs trimmed at the hilum, and placed into a cold centrifuge tube.

The two lungs were then homogenized (Janke and Kunkel Ultraturrax) with an equal volume of water. A sample of this homogenate was then added to homogenizing buffer to prepare a lung supernatant preparation (see chapter 3). The

remainder of the homogenate was used for the calculation of a wet/dry lung weight ratio (see chapter 2). The lung supernatant preparation was assayed for Na-K-ATPase activity (see chapter 3) and protein and DNA concentrations (see chapter 2). Na-K-ATPase activity was normalized to DNA because the protein concentrations of the lung supernatant samples of the instilled rats were significantly higher compared to those of the control rats due to the addition of protein derived from the instillate.

5.2.3 Determination of the rate of lung liquid clearance

The rate of lung liquid clearance in the above instilled rats was calculated by a modification of previously described methods (18,175,176). In brief, the dry lung weight (dQl) was first corrected for the mass of protein derived from instillate remaining in the lung after 45 minutes of ventilation. The mass (Q_{inst}) and fractional water content (FW_{inst}) of the instillate were known, and the fraction of instillate protein remaining in the lung after 45 minutes was taken as 0.870. This fraction was based on similar studies where (^{125}I)-albumin was used as a protein tracer (unpublished data). (^{125}I)-albumin was not used in these studies to avoid isotope crossover during scintillation counting for the Na-K-ATPase assay (177). Thus, the equation used for correction of dQl was:

$$dQl_{corr} = dQl - (Q_{inst} \times (1 - FW_{inst}) \times 0.870)$$

Lung liquid clearance was then calculated as:

$$\underset{(ml/hr)}{\text{Lung Liquid Clearance}} = (\underset{(ml)}{Q_{wl}(init)} - \underset{(ml)}{Q_{wl}(final)}) \;/\; \underset{(hrs)}{\text{time}}$$

$Q_{wl}(init)$ - Initial volume extravascular lung water

$Q_{wl}(final)$ - Final volume extravascular lung water

The initial volume of extravascular lung water ($Q_{wl}(init)$) was calculated by adding the volume of the instillate (Q_{inst}) to the volume of lung water in uninstilled rat lungs, estimated by multiplying the mean wet/dry lung weight ratio of the uninstilled control rats (3.697) by dQl_{corr}:

$$Q_{wl}(init) = Q_{inst} + (dQl_{corr} \times 3.697)$$

The final volume of extravascular lung water ($Q_{wl}(final)$ was derived from the standard wet/dry ratio calculations (see chapter 2).

5.2.4 Statistics

The data from the in vitro studies was analyzed by one-way ANOVA. Estimations of power and minimum detectable difference for this analysis were calculated as described (178). Changes in mean arterial and peak airway pressures, temperature, and arterial blood gas values between groups and between baseline and experimental periods within each group were analyzed by two-way ANOVA and the Fischer protected least significant difference (PLSD) test (137). The data on Na-K-ATPase activity and DNA recovery between control and instilled animals were analyzed using an unpaired t-test.

5.3 RESULTS

5.3.1 in vitro effects of thiourea on lung Na-K-ATPase activity

Figure 5.1 demonstrates the effect of thiourea on lung supernatant preparation Na-K-ATPase activity in vitro. Compared to the controls, thiourea had no significant effect on Na-K-ATPase activity at either of the concentrations tested ($p=0.58$, one-way ANOVA). This analysis had a power of 80%, with alpha set at 0.05, to detect a minimum difference of 51% from the mean control activity. Therefore, any potential in vitro effects of thiourea on lung Na-K-ATPase could not account for the increases in enzyme activity seen 8, 12, or 48 hours after thiourea treatment, as the increases in activity seen at these time points were all greater than 100% over the control values (figure 4.5).

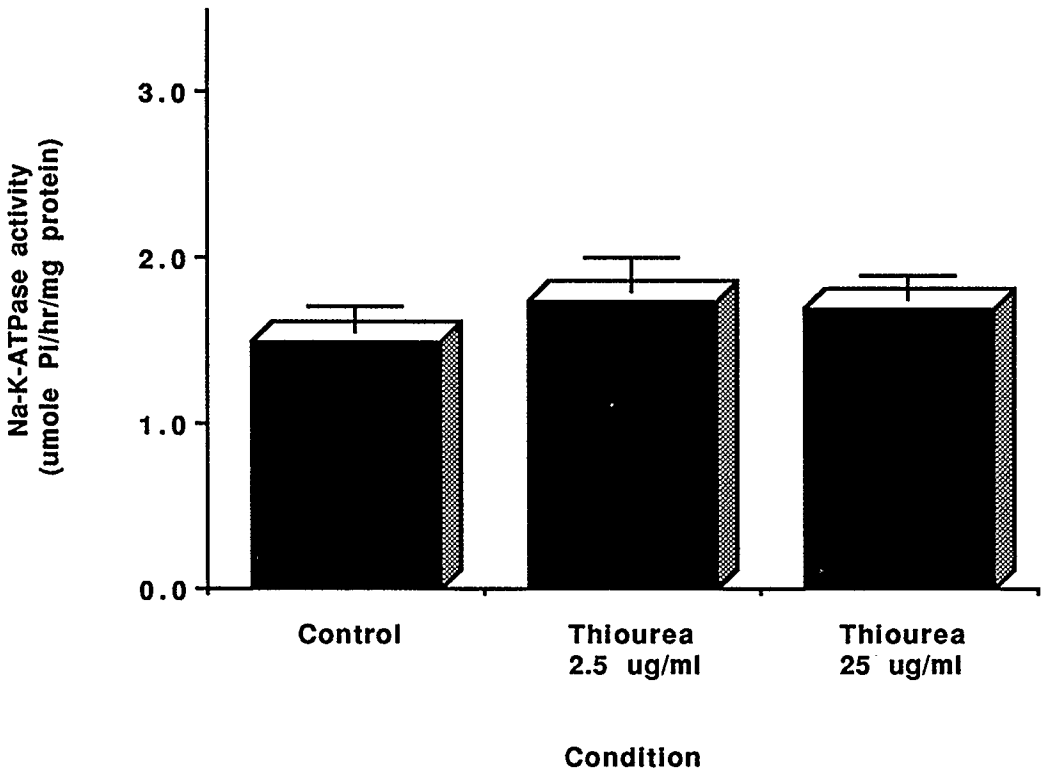


Figure 5.1 Effect of thiourea on lung supernatant preparation Na-K-ATPase activity *in vitro.* Lung supernatant was assayed in the presence or absence (control) of the indicated concentrations of thiourea. Thiourea had no effect on lung supernatant Na-K-ATPase activity *in vitro* (p=NS, one-way ANOVA).

5.3.2 Effects of alveolar flooding on lung Na-K-ATPase activity

The mean Na-K-ATPase activity of the uninstilled (control) rats was 25.5 +/- 6.6 umole Pi/hr/mg DNA, which was not significantly different from the mean activity of the saline-treated rats from the previous section (17.1 +/- 1.9 umole Pi/hr/mg DNA - figure 4.5) (p=0.11, unpaired t-test). Instillation of a protein-containing saline solution into the lungs resulted in a significant increase in lung supernatant Na-K-ATPase activity compared to the controls (figure 5.2) (p=0.027, unpaired t-test). This was not due to a difference in lung DNA content, as the recovery of DNA was similar between the instilled and control groups (3.4 +/- 0.3 versus 3.8 +/- 0.2 mg, p=0.36, unpaired t-test). The instilled rats cleared liquid at a mean rate of 1.04 +/- 0.04 ml/hr, which is similar to rates previously reported in rats (9,179), as well to rates obtained by our laboratory (unpublished data). There was no correlation between the rate of lung liquid clearance and Na-K-ATPase activity (r=0.32, p=0.54).

Relative to the control group, the instilled rats had significantly lower mean P_aO_2, higher mean P_aCO_2, and lower mean pH values during the experimental periods (table 5.1). Mean arterial and peak airway pressures and body temperature were similar between the two groups (table 5.2).

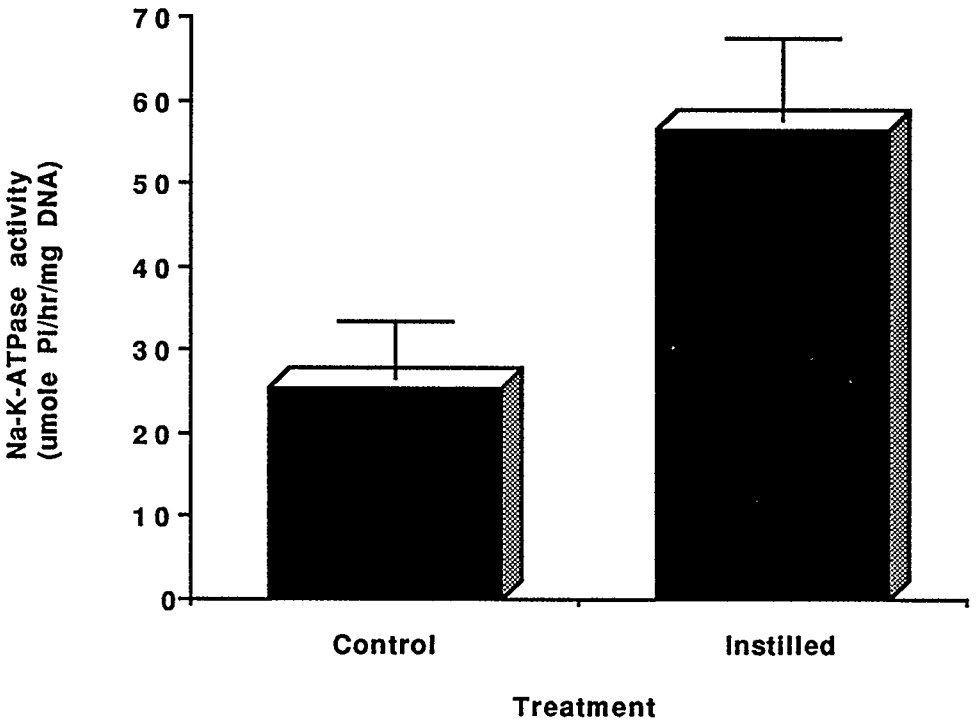


Figure 5.2 Na-K-ATPase activity in lung supernatant preparation from instilled and control rats. Na-K-ATPase activity was assayed in lung supernatant preparations derived from rats ventilated for 45 minutes after either being instilled with BSA-saline solution (instilled) or undergoing sham preparation (controls). The instilled group had a significantly higher mean Na-K-ATPase activity ($p=0.027$, unpaired t-test).

TABLE 5.1
ARTERIAL BLOOD GAS VALUES DURING VENTILATION OF INSTILLED AND CONTROL RATS.

	PaO_2		
Condition	Baseline	25 minutes[a]	45 minutes[a]
	mmHg	mmHg	mmHg
Controls	232+/-5	223+/-5	232+/-9
Instilled	248+/-11	81+/-6*+	88+/-8*+
	$PaCO_2$		
	Baseline	25 minutes[a]	45 minutes[a]
	mmHg	mmHg	mmHg
Controls	33.8+/-0.8	33.9+/-1.2	38.3+/-0.7*
Instilled	29.5+/-1.3	40.2+/-2.6*+	42.5+/-3.4*
	pH		
	Baseline	25 minutes[a]	45 minutes[a]
Controls	7.36+/-0.01	7.39+/-0.01	7.39+/-0.01
Instilled	7.39+/-0.01	7.31+/-0.01*+	7.32+/-0.02*+

a - time after instillation or equivalent period of ventilation in controls.
* - $p<0.05$ versus baseline value for respective condition.
\+ - $p<0.05$ versus control value at the same time point.

TABLE 5.2
MEAN ARTERIAL PRESSURE, PEAK AIRWAY PRESSURE, AND TEMPERATURE DURING VENTILATION OF INSTILLED AND CONTROL RATS.

	Mean Arterial Pressure		
Condition	Baseline	25 minutes[a]	45 minutes[a]
	mmHg	mmHg	mmHg
Controls	86.2+/-5.3	78.1+/-2.4	77.7+/-2.1
Instilled	83.1+/-3.0	77.8+/-3.0	76.8+/-3.6
	Peak Airway Pressure		
	Baseline	25 minutes[a]	45 minutes[a]
	CmH_2O	CmH_2O	CmH_2O
Controls	12.7+/-0.5	12.7+/-1.0	12.6+/-1.1
Instilled	13.8+/-0.7	14.4+/-1.0	14.4+/-1.1
	Temperature		
	Baseline	25 minutes[a]	45 minutes[a]
	^oC	^oC	^oC
Controls	36.4+/-0.2	37.1+/-0.1*	37.3+/-0.2*
Instilled	36.4+/-0.1	37.2+/-0.2*	37.1+/-0.1*

a - time after instillation or equivalent period of ventilation in controls.
* - $p<0.05$ versus baseline value for respective condition.

5.4 DISCUSSION

The results of this section provide two lines of evidence which suggest that the observed activation of lung Na-K-ATPase during the resolution of pulmonary edema was not likely due to an effect of thiourea on the enzyme. First, thiourea had no significant effects on lung Na-K-ATPase activity when applied in vitro, suggesting thiourea does not directly effect this enzyme. Second, the creation of alveolar flooding, in the absence of thiourea, activated Na-K-ATPase to a similar degree as was observed during the recovery from thiourea-induced pulmonary edema. Together this suggests that, in the setting of pulmonary edema, lung Na-K-ATPase activation is not dependent on the presence of thiourea.

A direct effect of thiourea on lung Na-K-ATPase was conceivable as radiolabeled thiourea has been demonstrated to bind to unidentified lung macromolecules (160,172-174). Although the absence of an effect of thiourea on Na-K-ATPase in vitro made any functionally significant direct thiourea-Na-K-ATPase interactions unlikely, indirect interactions were still possible. Indirect interactions could involve the action of activated metabolites of thiourea on Na-K-ATPase or the action of either thiourea or thiourea-derived metabolites on factors regulating Na-K-ATPase activity, such as the adenylate cyclase-cAMP system (18,69,77-79). There is evidence that thiourea, and other toxic thioureas, may

require biotransformation prior to exerting their toxic effects (161,172-174). This transformation may include oxidation to cyanide compounds (162), oxidation to atomic sulfur (161), or the creation of toxic oxygen free radicals (163). These metabolites have the potential to alter protein structure, and thus, function. For example, atomic sulfur may act to form hydrodisulphide moieties on cysteine amino acids (161). Transformation of thiourea occurs in the lung (161), possibly via interaction with proteins present in lung supernatant fractions (173). Although it is possible that the in vitro studies performed here may have allowed the biotransformation of thiourea, the temperature of incubation of lung sample with thiourea ($0^{o}C$) may not have been adequate. Incubation was performed at this relatively low temperature in order to minimize proteolytic damage to Na-K-ATPase. The 60-minute duration of exposure of Na-K-ATPase to thiourea in vitro was reasonable as in the in vivo lung injury model, significant pulmonary edema was observed 2 hours after thiourea injection (figure 4.1). Within these 2 hours, thiourea must undergo absorption, distribution, and lung accumulation, prior to exerting its cellular effects, all of which are bypassed with the in vitro exposure of Na-K-ATPase to thiourea. Thus, given the uncertainties with regard to the mechanisms of thiourea toxicity and the factors regulating Na-K-ATPase in the lung, the in vitro studies performed here could not rule out

indirect effects of thiourea that may be modulating Na-K-ATPase activity.

As the above in vitro studies could not account for potential indirect effects of thiourea on lung Na-K-ATPase, another approach to demonstrate that thiourea was not likely mediating the effects on Na-K-ATPase activity seen during edema recovery was needed. The second approach that was adopted was to demonstrate Na-K-ATPase activation utilizing a different model of pulmonary edema. In this study, a well characterized model of alveolar flooding was used, whereby a solution, designed to mimic the fluid present in the airspaces during edema secondary to lung injury (180), was instilled into the lungs of ventilated rats. After 45 minutes of ventilation, the instilled rats had significant activation of Na-K-ATPase relative to sham controls. This activation of Na-K-ATPase occurred during a time when liquid was being cleared from the lungs, mimicking the relationship between the time courses of edema recovery and Na-K-ATPase activation seen using the thiourea model of edema (figure 4.7). As the instilled and control groups were treated in the same manner except for fluid instillation, the observed activation cannot be attributed to differences in anesthesia, surgery, ventilation, or physiologic support. Further, there were no significant differences in mean arterial or peak airway pressures or in temperature between the instilled and control groups (table 5.2). The

differences observed between the two groups in PaO_2 and $PaCO_2$ are expected given the intrapulmonary shunting created with extensive alveolar flooding (181).

There are many possible mediators for the observed activation of lung Na-K-ATPase in the alveolar flooding model utilized in this study. The previously mentioned neurohormonal changes associated with lung injury (see chapter 1 - summary), may be active, although hypoxemia-induced release of ADH (96) is not likely to be a major factor, as the instilled rats did not become significantly hypoxemic (table 5.1). Changes in ambient pH may affect Na-K-ATPase activity (182). In this study, the instilled group had significantly lower systemic arterial pH values compared to the controls (table 5.1). As well, the instilled group had significantly higher arterial CO_2 tensions (table 5.1), suggesting the acidemia was partially respiratory in origin. When the instilled and control groups were analyzed together, there was a significant negative correlation between arterial pH and lung Na-K-ATPase activity (25 minutes: $r=0.76$, $p<0.01$; 45 minutes: $r=0.82$, $p<0.001$) and a significant positive correlation between arterial CO_2 tension and Na-K-ATPase activity (25 minutes: $r=0.68$, $P<0.02$; 45 minutes: $r=0.67$, $P<0.02$). This is an interesting observation as an association between altered renal sodium excretion and elevated arterial CO_2 tensions has been noted in patients with chronic obstructive airways disease (183-

185). These hypercarbic patients had significantly increased circulating levels of aldosterone and ADH (184,185), both of which are known activators of Na-K-ATPase (68,74) and lung sodium transport (81-83). The association between acidemia and sodium handling has not been well defined. Changes in pH could have direct or indirect effects, via local or systemic hormonal alterations, on Na-K-ATPase. Further study into the relationship between arterial PCO_2 and pH and lung Na-K-ATPase and lung sodium transport is clearly warranted.

Changes in alveolar inflammatory cell number or specific activity may contribute to changes in total lung Na-K-ATPase activity. Although not specifically examined here, in a previous investigation using a similar model of alveolar flooding in spontaneously breathing rats, there were no significant changes in the number of BAL inflammatory cells 4 hours after instillation (90). Alveolar inflammatory cell accumulation would be expected to be even less with this study, as the animals were only ventilated for 45 minutes. Blood-derived cellular elements likely do not contribute significantly to changes in whole lung Na-K-ATPase activity either, as, similar to the in vivo thiourea study (chapter 4), the animals were exsanguinated prior to examination, and there was no correlation between the subsequent mass of blood in the lungs and Na-K-ATPase activity ($r=0.35$, $p=NS$).

In summary, these studies suggest that thiourea is not necessary for the activation of lung Na-K-ATPase during the resolution of lung edema. In combination with the demonstrated lack of in vitro effects of thiourea on lung Na-K-ATPase, this suggests that it is the presence of edema, and/or the physiologic consequences of edema, that mediates the changes in Na-K-ATPase activity during edema recovery.

CHAPTER 6

THE CONTRIBUTION OF ALVEOLAR INFLAMMATORY CELLS TO CHANGES IN TOTAL LUNG Na-K-ATPase ACTIVITY DURING THE RECOVERY FROM THIOUREA-INDUCED PULMONARY EDEMA

6.1 INTRODUCTION

Alveolar inflammatory cells, predominantly neutrophils, have been noted to accumulate in the airspaces of humans suffering from lung injury (168), as well as in several animal models of high-permeability pulmonary edema (11,169), including that induced by the thiourea ANTU (170). An increase in either the number of alveolar inflammatory cells and/or the specific Na-K-ATPase activity per cell could potentially account for the increase in total lung Na-K-ATPase activity observed during the resolution from thiourea-induced pulmonary edema (see chapter 4). In order to assess this possibility, the objective of this section was, therefore, to evaluate both the number and Na-K-ATPase activity of alveolar inflammatory cells obtained by broncho-alveolar lavage (BAL) during the recovery from thiourea induced pulmonary edema.

6.2 METHODS

6.2.1 Protocol

To determine the contribution of alveolar inflammatory cells to changes in whole-lung Na-K-ATPase activity during recovery from thiourea-induced pulmonary edema, the activity of Na-K-ATPase was measured in cells obtained by broncho-alveolar lavage (BAL) from rats treated with either thiourea (n=5) or saline (n=5). The animals were examined 12 hours

after injection, this being when maximal activation of total lung Na-K-ATPase was seen (figure 4.5). The relative contribution of BAL cell Na-K-ATPase activity to total lung activity was estimated by dividing the total Na-K-ATPase activity of the BAL cells (activity/cell x total number of cells) by the total lung Na-K-ATPase activity 12 hours after treatment (activity/mg DNA x total DNA recovery).

6.2.2 Broncho-alveolar lavage

Under pentobarbital anesthesia (60 mg/Kg IP), the animals were exsanguinated via transection of the abdominal aorta and inferior vena cava. A tracheostomy was performed and a blunt 16-gauge needle cuffed with plastic tubing was inserted into the trachea and secured with ties. The diaphragm was then punctured and lavage performed with sterile normal saline in 5 ml aliquots for a total of 50 ml. The lavage was stored on ice until use. A 0.1 ml aliquot of the lavage was used to determine a total cell count using a hemocytometer (Reichert-Jung). A second 0.1 ml aliquot was cytocentifuged onto a glass slide, air dried, and then stained using a modified Wright's preparation (LeukoStat Staining Kit, Fisher Scientific) to obtain a differential cell count. A random sample of at least 100 cells was used.

The remaining cell suspension was centrifuged at 200g x 10 min at $4^{O}C$. The resulting cell pellet was resuspended in 350ul of homogenization buffer by sonication (2 x 7.5 seconds - 75 Watts), and then assayed for protein

concentration (see chapter 2) and Na-K-ATPase activity. The supernatant from the centrifuged lavage was assayed for protein concentration (see chapter 2) in order to confirm the high-permeability nature of the lung injury induced by thiourea.

6.2.3 Na-K-ATPase activity of BAL cells

Na-K-ATPase activity was determined using a modification of the technique used for lung supernatant preparation. The assay conditions used were as previously described (186): NaCl 50mM, KCl 5mM, MgCl2 7.5mM, ATP 4mM with 5-10 uCi gamma ^{32}P-ATP (ICN Biomedicals) per sample, EGTA 1mM, sodium azide 5mM, Imidazole 25mM pH 7.40 (37^{O}C), with or without ouabain 2mM. No further pre-treatment permeabilization steps were utilized as both ionic and nonionic detergents have been reported to significantly inhibit pulmonary alveolar macrophage Na-K-ATPase activity (186). The procedure began by adding 150 ul (50-100 ug of protein) of BAL sonicate to 750ul of assay buffer, with and without ouabain. The tubes were then incubated for 20 minutes on ice followed by 10 minutes at 37^{O}C prior to initiating the assay by the addition of ATP. Aliquots of 0.1 ml were then sampled from each tube at 3-minute intervals for a total of 24 minutes, and placed into filtration columns. The remainder of the assay and analysis was as described in chapter 3. Only one assay per sample was possible due to the limited amount of protein. Na-K-

ATPase activity was normalized to cell count as well as to protein, as measured in the sonicated preparation of BAL cells.

6.2.4 Statistics

Differences in total and differential cell counts and Na-K-ATPase activity of the BAL cells between the thiourea and saline (control) groups were analyzed using unpaired t-tests.

6.3 RESULTS

There was no significant difference in the number of inflammatory cells recovered by BAL between the saline and thiourea groups (table 6.1) ($t=1.2$, $p=0.26$). Further, there were no significant differences in the total number of macrophages or neutrophils recovered by BAL between the two groups (table 6.1).

Mean cellular Na-K-ATPase activity in the BAL samples did not differ significantly between the thiourea and saline treated animals ($t=0.18$, $p=0.87$) (table 6.2). Similarly, the total BAL Na-K-ATPase activity did not differ between the two groups ($t=0.23$, $p=0.82$) (table 6.2). BAL Na-K-ATPase activity accounted for only 0.010+/-0.002% of total lung Na-K-ATPase activity in the thiourea-treated animals, and 0.034+/-0.007% of total lung Na-K-ATPase activity in the saline treated animals.

Thiourea-treated animals had a significantly higher concentration of protein in the BAL supernatant compared to the saline-treated animals (0.311+/-0.055 versus 0.033+/-0.002 mg/ml, $t=5.0$, $p<0.01$).

TABLE 6.1
TOTAL AND DIFFERENTIAL CELL COUNTS OF ALVEOLAR INFLAMMATORY CELLS RECOVERED BY BRONCHO-ALVEOLAR LAVAGE 12 HOURS AFTER TREATMENT.

Group	Total Cell Count ($\times 10^6$)	Macrophages ($\times 10^6$)	Neutrophils ($\times 10^6$)	Lymphocytes ($\times 10^6$)
Thiourea (n=5)	3.4+/-0.6	3.3+/-0.6	0.10+/-0.03	0.02+/-0.01
Saline (n=5)	2.6+/-0.2	2.6+/-0.2	0.03+/-0.01	0.03+/-0.01

TABLE 6.2
TOTAL AND CELLULAR Na-K-ATPase ACTIVITY OF ALVEOLAR INFLAMMATORY CELLS RECOVERED BY BRONCHO-ALVEOLAR LAVAGE 12 HOURS AFTER TREATMENT.

Group	Cellular Na-K-ATPase Activity (umole Pi/hr/10^9 cells)	Total BAL Na-K-ATPase Activity* (umole Pi/hr)
Thiourea (n=5)	8.0+/-2.2	24.2+/-4.3
Saline (n=5)	8.5+/-1.8	22.7+/-4.8

* - Total BAL Na-K-ATPase activity = activity/cell x total number of cells.

6.4 DISCUSSION

Treatment with thiourea failed to significantly increase either mean alveolar inflammatory cell number or Na-K-ATPase activity. As a result, total BAL Na-K-ATPase activity also did not change significantly after treatment with thiourea relative to saline-treated controls. Together, this data indicates that the observed increase in total lung Na-K-ATPase activity during the resolution of thiourea induced pulmonary edema cannot by explained by changes in alveolar inflammatory cells.

The statistically insignificant increase in alveolar inflammatory cell number observed in this study after treatment with thiourea was not as dramatic as seen previously. Day et al (170) found an approximately three fold increase in the number of BAL white blood cells 8 hours after treating Sprague-Dawley rats with 3.5 mg/Kg ANTU (equivalent to approximately 1.3 mg/Kg of thiourea on an equimolar basis) compared with controls, this despite using a less rigorous BAL procedure (2 x 7 ml aliquots). This difference may be related to unknown differences between the pulmonary effects of thiourea and ANTU, or may be related to the difference in time after treatment when the BAL was performed. In this study, BAL was performed 12 hours after treatment, this being when maximal changes in lung supernatant preparation Na-K-ATPase activity were seen after thiourea treatment (figure 4.5). Although not specifically

examined in this study, it is unlikely that BAL cells contribute significantly to total lung Na-K-ATPase activity at other time points after thiourea treatment given that the proportion of BAL cell/total lung Na-K-ATPase activity was very low (<0.1%) in both the saline and thiourea groups.

The minor increase in mean BAL cell number observed in this study after thiourea treatment was composed primarily of macrophages (table 6.1). Neutrophils, although increasing three-fold, comprised a relatively small proportion of the total BAL cellular population. Neither of these differences were statistically significant. In studies of human ARDS patients, the majority of cells accumulating in the alveolar spaces and BAL fluid have been neutrophils (168). Similarly, in many animal models of lung injury, neutrophils have been the predominant cell type accumulating acutely in the airspaces (169,187). However, significant increases in the number of BAL macrophages have been found in human victims of smoke inhalation (188), in rats sustaining lung injury via the instillation of intra-tracheal IgA (189), and in sheep instilled with autologous serum (187). There are, unfortunately, no other studies examining changes in specific BAL cell types in response to thioureas.

Na-K-ATPase activity was evaluated in sonicated BAL cells using previously described conditions (186) to allow for comparison between studies. When normalized to protein,

the mean activity of the BAL preparation used in this study was very similar to that obtained by Mustafa et al (186) using sheep pulmonary alveolar macrophages (1.84 vs 1.75 umole Pi/hr/mg protein, saline controls vs sheep macrophages (186)). Unfortunately, there are no similar studies using rats. Total BAL Na-K-ATPase activity did not differ significantly between the thiourea and saline groups. Further, BAL activity comprised a miniscule proportion of the total lung Na-K-ATPase activity (<0.1% in both groups). This is not surprising, however, as it has been demonstrated that macrophages, located primarily in the alveolar region, comprise only 3% of total cells in normal rat lungs (47). Although it would be desirable to perform similar analysis on other specific lung cell types in order to further localize changes in Na-K-ATPase activity, a lack of reliable techniques for isolation of these cell types largely precludes this (see chapter 1).

Consistent with an increased protein permeability induced by thiourea, BAL protein concentrations were significantly higher in treated animals relative to controls. Unfortunately, differences in BAL techniques, particularly with regards to the total lavage volumes utilized, make comparison with other studies difficult (168,170,190).

In summary, this study demonstrates that the observed increase in total lung Na-K-ATPase activity occurring after

treatment with thiourea cannot be explained by changes in alveolar inflammatory cell number or specific enzyme activity. Further localization of the changes in Na-K-ATPase activity occurring during edema resolution becomes difficult, largely due to the limited ability to reliably isolate specific lung cellular components.

CHAPTER 7

QUANTITATION OF LUNG Na-K-ATPase PROTEIN DURING THE RESOLUTION FROM HIGH-PERMEABILITY PULMONARY EDEMA

7.1 INTRODUCTION

In chapter 4 it was demonstrated that the activity of lung Na-K-ATPase increased during the resolution of pulmonary edema. There are 2 broad biochemical mechanisms by which this increase in activity could occur: the number of enzyme units in the lung could increase and/or the molecular activity (turnover rate) of existing enzyme units could increase. Both of these mechanisms have been demonstrated to be active in the regulation of Na-K-ATPase in AIIC during different stages of lung development (93). Furthermore, Nici *et al* (94) noted an apparent increase in the amount of Na-K-ATPase protein in lung after hyperoxic injury. Although in the study by Nici *et al* no concurrent measurement of Na-K-ATPase activity was performed, this study suggests that regulation of lung Na-K-ATPase protein expression could potentially contribute to the regulation of lung Na-K-ATPase activity.

An examination into the general biochemical mechanisms by which lung Na-K-ATPase activity is modulated is important in order to obtain an initial understanding of the specific factors potentially regulating lung Na-K-ATPase and compare these to factors regulating overall lung sodium transport. If changes in the number of lung Na-K-ATPase enzyme units underlies changes in lung Na-K-ATPase activity, then factors

influencing Na-K-ATPase gene expression, such as aldosterone (68,191), would likely be active in the lung, whereas if changes in the molecular activity of existing Na-K-ATPase enzyme units underlies changes in lung Na-K-ATPase activity, then factors influencing the Na-K-ATPase protein itself, such as cAMP (76-78), would more likely be active in the lung (see chapter 1, section 2b - regulation of Na-K-ATPase activity for further information).

In order to address the possible mechanisms underlying changes in lung Na-K-ATPase activity, the objective of this section was to determine whether the quantity of Na-K-ATPase enzyme in the lung changes during the resolution from increased permeability pulmonary edema.

7.2 METHODS

7.2.1 Protocol

To determine whether the quantity of Na-K-ATPase enzyme in the lung changes during the resolution from high permeability pulmonary edema, the amount of Na-K-ATPase alpha subunit was quantified in lung supernatant preparations derived from rats treated with either thiourea or saline (controls). Rats were injected with either thiourea (1.5 mg/Kg IP) or an equivalent volume of normal saline and sacrificed 12 or 48 hours later (n=5 rats at each time point for each treatment). The 12-hour time point was examined as this is when total lung Na-K-ATPase activity was maximal after thiourea treatment (figure 4.5). The 48-hour

time point was examined to account for new enzyme synthesis that may have been delayed. Lung supernatant preparations were prepared as previously described (see chapter 3). Na-K-ATPase alpha-1 subunit was quantified using the immunoblot procedure, with reference to known quantities of Na-K-ATPase standards that were run concurrently.

7.2.2 Purification of Na-K-ATPase Standards

Na-K-ATPase was partially purified from rat kidney following previously described procedures (121,192). As rat kidney expresses predominantly the alpha-1 isoform of the Na-K-ATPase, it is a suitable standard for comparison with lung samples (55,63). Kidney was chosen over lung tissue for partial purification of a Na-K-ATPase standard as the yield of enzyme from kidney is much higher. All procedures were performed at $4^{O}C$. In brief, 7 adult male Sprague-Dawley rats were sacrificed and their kidneys removed. The outer medulla of the kidneys was carefully dissected away and placed into cold sucrose buffer (sucrose 0.25M, EDTA 1mM, Imidazole 25mM - pH 7.2). This was then homogenized (Polytron) on ice for 3 x 20 second bursts. The homogenate was then centrifuged (6000g, 15 minutes, $4^{O}C$) and the resulting supernatant was collected and saved. The pellet was re-homogenized in sucrose buffer and centrifuged as before. The resulting supernatant was collected and added to the first supernatant to yield a combined supernatant (S1). This combined supernatant was then centrifuged

(48000g, 30 minutes, 4^{O}C), the resulting supernatant (S2) collected, and the pellet was resuspended in imidazole buffer (EDTA 2mM, imidazole 50mM - pH 7.5) to yield a microsomal fraction (M). The microsomal fraction was then incubated with sodium dodecyl sulphate (SDS) for 45 minutes at room temperature in order to solubilize the majority of membrane proteins while leaving Na-K-ATPase largely within the membranes (121,192). The incubation conditions were: protein concentration 1.4 mg/ml, SDS 0.57 mg/ml, ATP 3mM, EDTA 2mM, Imidazole 50mM - pH 7.5. Discontinuous sucrose density gradients, consisting of 9.5 ml of 25%, 5.7 ml of 15%, and 3 ml of 10% (w/v) sucrose in EDTA 1mM, imidazole 25mM - pH 7.5, were prepared in 2.5 x 9 mm Ti60 (Beckman) centrifuge tubes. 7.5 ml aliquots of the SDS incubation mixture were layered on each of the gradients and these were then centrifuged (Beckman Ti60 rotor, 50000 rpm, 150 minutes, 4^{O}C). The resulting supernatant (S3) was removed and the remaining pellets, containing partially purified Na-K-ATPase enzyme (NaK), were resuspended in sucrose buffer and stored at -70^{O}C prior to use.

Na-K-ATPase activity was determined for the microsomal and partially purified Na-K-ATPase fractions, after pre-treatment with deoxycholic acid, as described (121).

The electrophoretic purity of the partially purified Na-K-ATPase sample was determined by performing scanning densiometry on stained gels of the Na-K-ATPase standards.

Gel electrophoresis was performed as described under Immunoblotting Procedure. A photograph of the gel was scanned (Abacon Scanner) and analyzed for two-dimensional densiometric area (Scan Analysis program, Biosoft, UK). The alpha-subunit band was identified by comparison with an immunoblot of a concurrently run identical gel (prepared as described under Immunoblotting Procedure). The beta-subunit band was identified as a prominent peak with a molecular mass of approximately 50 KDa (2,5). The fraction of alpha subunit in the Na-K-ATPase standard was taken as the densiometric area of the alpha subunit band/total densiometric area for the entire gel lane, with 4 analyses of different gel lanes being averaged to obtain the final fraction.

7.2.3 Immunoblotting Procedure

Immunoblotting was the procedure used to identify and quantify the alpha subunit of the Na-K-ATPase in the lung samples. The immunoblotting procedure was begun immediately after obtaining the lung supernatant preparation for each rat, and was completed without interruption, so as to avoid freezing and potential denaturation of the samples, as was observed in several preliminary experiments.

5-ml aliquots of lung supernatant preparation from each rat were first centrifuged (100,000g, 1 hour, $4^{o}C$) to form a pellet in order to concentrate the membrane proteins. The pellet was resuspended in 350 ul of homogenization buffer

containing protease inhibitors (see chapter 3) by sonication (2 x 10 second bursts - 75 Watts) on ice to yield a crude membrane fraction. Protein concentration was measured as previously described (see chapter 2).

Triplicate 300 ug samples of the crude membrane fraction derived from each rat were then subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE), along with known amounts of Na-K-ATPase standard (1.29, 1.72, 2.16, 2.59, 3.45, 4.31 ug). Na-K-ATPase standards were run on each gel to act as internal controls for quantitation of enzyme amount. Samples from both thiourea and saline treated rats were run on each gel. SDS-PAGE was performed in 1.5 mm thick 7.5% polyacrylamide separating gels with 4.0% polyacrylamide stacking gels, in the presence of 0.1% (w/v) SDS, using the discontinuous buffer system of Lammeli (193). SDS-PAGE was performed at room temperature using a constant current of 25mA through the stacking gel and 35mA through the separating gel. Protein samples were diluted 1:1 with sample buffer and incubated for 30 minutes at room temperature prior to gel loading, so as to avoid hydrolysis of Na-K-ATPase (194,195). The sample buffer, after dilution with the protein, consisted of: SDS 2% (w/v), beta-mercaptoethanol 5% (v/v), glycerol 10% (v/v), bromophenol blue 0.01% (w/v), and Tris 62.5mM - pH 6.8.

The electrophoresed proteins were transferred to nitrocellulose membranes (0.2 uM) (Transblot, BioRad) at a

constant current of 200 mA for 4 hours at $4^{o}C$ (196). The residual gel was routinely stained with Coomassie Blue after blotting to ensure protein transfer. Proteins <200 KDa consistently transferred completely, whereas proteins >200 KDa did not. In order to ensure adequate retention of Na-K-ATPase by the nitrocellulose membrane, on 2 occasions a second membrane was placed immediately adjacent to the primary membrane prior to transfer and was subsequently examined for Na-K-ATPase protein as described below. No Na-K-ATPase protein was detected in either of these trials.

The remaining procedures were carried out at room temperature. The resulting blot was blocked overnight in 5% (w/v) non-fat milk powder, 0.5% (v/v) Tween-20, 0.01% (w/v) sodium azide in Tris-saline (NaCl 150mM, Tris 20mM - pH 7.5) under continuous agitation. The blot was then incubated with the primary antibody McK1, a mouse-IgG monoclonal antibody specific for the alpha-1 isoform of the Na-K-ATPase (195), diluted 1:50 in Tris-saline with 1% non-fat milk powder, 0.05% tween-20, and 0.01% sodium azide. This antibody was the kind gift of Dr. K. Sweadner, Harvard University. Incubation was for 1 hour under continuous agitation. The blot was then washed for 1 hour with 3 changes of Tris-saline with 0.05% Tween-20 and then incubated with secondary antibody (goat anti-mouse IgG alkaline phosphatase conjugate - Sigma) diluted 1:1200 in Tris-saline with 1% non-fat milk powder and 0.05% tween-20,

for 30 minutes under constant agitation. The blot was then washed as before. The bound second antibody was detected using a nitro-blue tetrazolium, 5-bromo-4-chloro-3-indoyl-phosphate developing system (BioRad). The blots were rinsed in several changes of water to stop the reaction once developed.

A control blot, incubated with non-specific mouse serum instead of the McK1 primary antibody, was also prepared to ensure that binding of the secondary antibody was specific.

The developed blots were scanned (Abacon Scanner) at maximum resolution and the two-dimensional densiometric area of the alpha subunit bands for the samples and concurrently run standards were obtained (Scan Analysis program, Biosoft, UK). The values were corrected for background. The 3 densiometric areas for each lung sample were averaged to yield a single value for each rat. Linear regression lines were then obtained for the Na-K-ATPase standards on a given blot relating the densiometric area and the amount of alpha subunit protein loaded (derived by multiplying the total amount of Na-K-ATPase standard loaded by the fraction of alpha subunit in the standard). The densiometric areas of the lung samples were then applied to the appropriate regression equation to obtain the amount of Na-K-ATPase alpha subunit. These values were then normalized to the equivalent amount of DNA loaded in each lane, as measured in the lung supernatant preparation (see chapter 2), to yield a

final value in the units of ug alpha-1 subunit/mg DNA for each rat.

7.2.4 Statistics

Linear regression lines for the densiometric absorbance of the Na-K-ATPase standards were obtained by standard methods (197). The recovery of DNA and the concentrations of Na-K-ATPase alpha subunit in lung supernatant between the saline and thiourea groups were compared using unpaired t-tests.

7.3 RESULTS

7.3.1 Partial purification of Na-K-ATPase standard

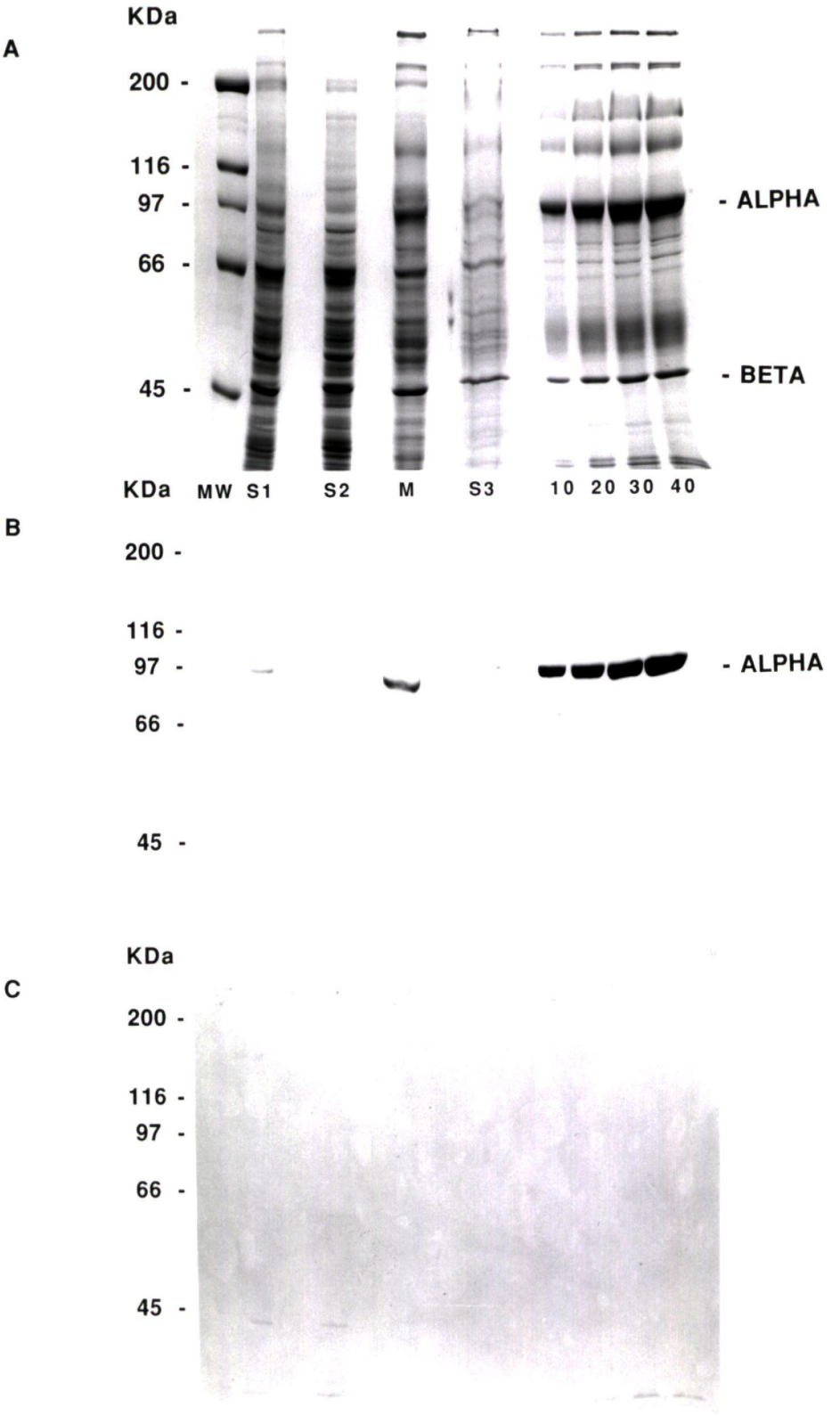
Na-K-ATPase was partially purified from the outer medulla of rat kidney. The final sample had a specific activity of 398 umole Pi/hr/mg protein. Previous investigators have obtained samples with activities of 500-700 umole Pi/hr/mg protein using similar procedures and animal species (146,198). Based on the ratio of enzyme activities, the final sample represented a 4.7 fold purification from the microsomal fraction. 91.5% of the ATPase activity was ouabain-inhibitable.

SDS-PAGE of samples from the various stages of purification are demonstrated in figure 7.1A. Two principle bands of M_r 50 and 97 KDa were identified in the final sample. The 97 KDa protein was identified as the alpha-1 subunit of Na-K-ATPase by reference to a concurrently run immunoblot of the purification samples (figure 7.1B). The 50 KDa protein likely represented the beta subunit of the Na-K-ATPase (192,194,195).

Scanning densiometry of the gel lanes containing the partially purified Na-K-ATPase in figure 7.1A indicated that the alpha subunit comprised 43 +/- 4% of the total protein in the sample. The alpha and beta subunits combined comprised 52 +/- 4% of the total protein, which is of comparable purity to that obtained previously using this method (121).

Figure 7.1 Purification of rat kidney Na-k-ATPase standards. Masses of molecular weight markers are given to the left of the gel and immunoblots. Lanes are identified between **A** and **B**: **MW** - molecular weight markers; **S1,S2,M,S3** - as described in methods; **10,20,30,40** - partially purified rat-kidney Na-K-ATPase in given amounts (ug) per lane.
A: SDS-PAGE of indicated samples. Labels indicate the expected locations of the alpha and beta subunits of Na-K-ATPase.
B: Immunoblot of same samples as in **A** stained with McK1 antibody which is specific for the alpha-1 subunit of Na-K-ATPase. Label points to the alpha-1 subunit of Na-K-ATPase.
C: Immunoblot of same samples in **A** stained with nonspecific mouse serum.

The McK1 antibody detected a single band of M_r 97 KDa. Nonspecific mouse serum failed to stain bands in the region of Na-K-ATPase alpha subunit suggesting that McK1 binding was specific.


A
KDa
200 -
116 -
97 -
66 -
45 -
- ALPHA
- BETA
KDa MW S1 S2 M S3 10 20 30 40
B
200 -
116 -
97 -
66 -
45 -
- ALPHA
C
KDa
200 -
116 -
97 -
66 -
45 -

7.3.2 Validation of immunoblot quantification of Na-K -ATPase

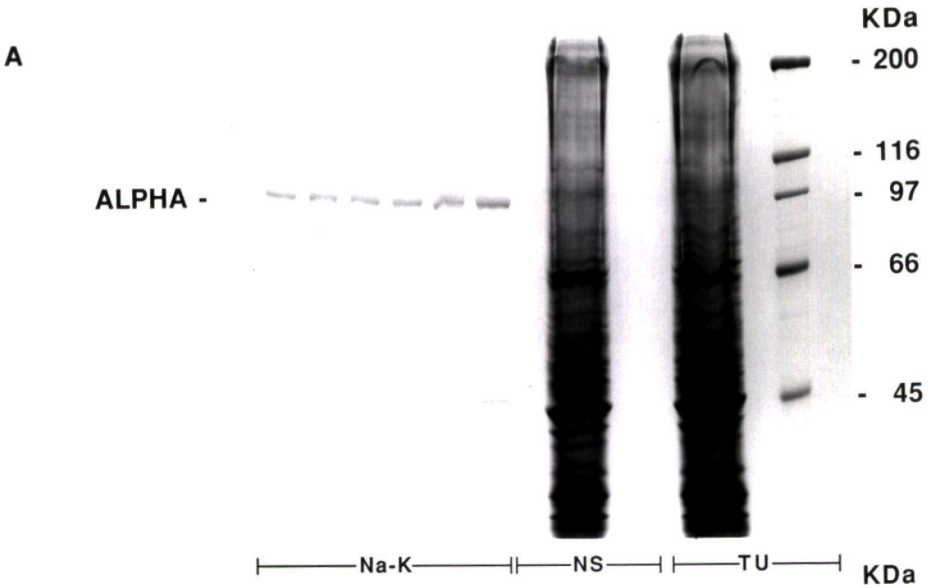
The immunoblot procedure allowed for the sensitive and specific detection of the alpha-1 subunit of Na-K-ATPase. This technique detected a single predominant band of molecular mass 97 KDa in both the kidney standards (figure 7.1B) and lung samples (figure 7.2B). Incubation with non-specific mouse serum resulted in weak staining of several bands of molecular weight <45 KDa, however, did not stain any bands in region of the Na-K-ATPase alpha subunit (figure 7.1C), suggesting that the binding of the McK1 antibody was specific. The lowest detectable quantity of Na-K-ATPase alpha subunit was 0.57 ug (figure 7.2B). The relation between the amount of Na-K-ATPase alpha subunit and densiometric area was linear up to 1.89 ug (figure 7.2C). Densiometric areas for the lung samples always fell within this linear range. Lung samples from rats treated with thiourea and saline produced clearly visible bands on the immunoblots which were reproducible over the 3 samples run for each rat.

Figure 7.2 Quantification of Na-K-ATPase alpha-1 subunit in lung supernatant preparation - representative example. Masses of molecular weight markers are given to the right of the gel and immunoblot. Lanes are identified between **A** and **B**: **NaK** - partially purified rat kidney Na-K-ATPase standards; **NS** - lung supernatant preparation from saline control (48 hours after treatment); **TU** - lung supernatant preparation from thiourea-treated rat (48 hours after treatment).

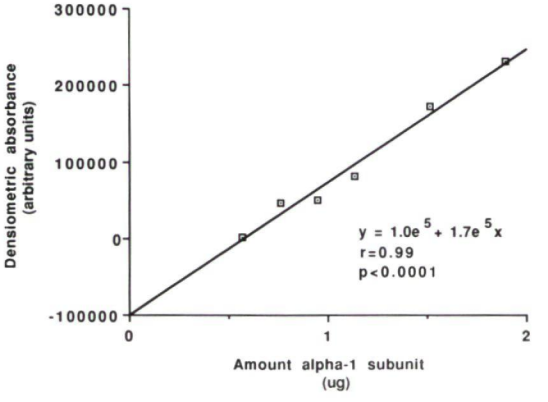
A: SDS-PAGE of Na-K-ATPase standards (0.57-1.89 ug) and lung supernatant preparation from saline- and thiourea-treated rats (300 ug/lane - single sample each rat). Rightmost lane contains molecular weight markers.

B: Immunoblot of same samples in **A** stained with McK1 antibody - triplicate samples of lung supernatant preparations from saline- and thiourea-treated rats (300 ug/lane).

C: Linear relationship between amount of rat kidney Na-K-ATPase alpha subunit and densiometric area.


A
KDa
- 200
- 116
- 97
- 66
- 45
ALPHA -
Na-K
NS
TU
KDa
B
- 200
- 116
- 97
- 66
- 45
ALPHA -

C


300000
200000
100000
0
-100000
Densiometric absorbance (arbitrary units)
y = 1.0e 5 + 1.7e 5 x
r=0.99
p<0.0001
0
1
2
Amount alpha-1 subunit (ug)

7.3.3 Quantification of lung Na-K-ATPase alpha subunit during edema resolution

The amount of Na-K-ATPase alpha subunit was significantly increased 12 and 48 hours after treatment with thiourea relative to saline controls (table 7.1). This difference was not due to changes in lung DNA content as the recovery of DNA was similar between the thiourea and control groups (table 7.2). The amount of Na-K-ATPase alpha subunit did not significantly differ between the 12- and 48-hour time points in either the thiourea or saline groups.

TABLE 7.1
CONCENTRATION OF Na-K-ATPase ALPHA SUBUNIT IN LUNG SUPERNATANT 12 AND 48 HOURS AFTER TREATMENT.

	Na-K-ATPase alpha subunit concentration (ug alpha-1 subunit/mg DNA)	
Treatment	12 hours	48 hours
Saline (n=5)	7.5+/-1.1	8.7+/-0.8
Thiourea (n=5)	15.2+/-2.6*	13.5+/-1.0*

* - $p<0.05$ versus control value at same time point.

TABLE 7.2
DNA RECOVERY 12 AND 48 HOURS AFTER TREATMENT.*

	DNA Recovery (mg)	
Treatment	12 hours	48 hours
Saline (n=5)	2.2+/-0.4	2.1+/-0.2
Thiourea (n=5)	1.8+/-0.2	1.9+/-0.2

* - there were no significant differences between the groups

7.4 DISCUSSION

This study demonstrated that the amount of Na-K-ATPase protein in the lung increased during the resolution from high-permeability pulmonary edema. This suggests that the observed increase in lung Na-K-ATPase activity may be mediated, at least partially, by increases in the number of Na-K-ATPase enzyme units in the lung.

Chapman et al (93) provided evidence for two potential mechanisms underlying changes in Na-K-ATPase activity in rat AIIC during different stages of development. Perinatally, it was found that AIIC Na-K-ATPase activity increased secondary to an increase in the molecular activity (turnover number) of pre-existing enzyme units whereas postnatally AIIC Na-K-ATPase activity increased secondary to an increase in the number of enzyme units per cell (93). By applying a ouabain-binding assay to the same cellular preparation used for activity measurements, direct quantitation of the number of Na-K-ATPase units per cell and subsequent calculation of turnover number was readily performed (93,199). An identical analysis, unfortunately, cannot be performed in these studies, as the enzyme activities and enzyme quantities were not measured in the same animals. An estimate of turnover number, however, can be obtained by relating the mean Na-K-ATPase activity to the mean Na-K-ATPase protein concentration for a given time point. This is valid as both the enzyme activity and quantity were

evaluated in lung supernatant preparations derived from the same sex, species and size of rats using identical methods of lung injury. As one is taking a ratio of means, no estimate of variability is obtained. Turnover number is calculated by dividing the mean Na-K-ATPase activity (figure 4.5) by the mean Na-K-ATPase quantity, after converting the enzyme quantity to umoles by dividing by the molecular mass of the alpha subunit of Na-K-ATPase (97 KDa) (199). As shown in table 7.3, the turnover number for lung Na-K-ATPase also increased during the resolution from thiourea-induced pulmonary edema. This would suggest that an increase in the molecular activity of pre-existing and/or newly synthesized Na-K-ATPase enzyme units may also contribute to the observed increase in total lung Na-K-ATPase activity during edema resolution.

TABLE 7.3
TURNOVER NUMBERS FOR LUNG Na-K-ATPase 12 AND 48 HOURS AFTER TREATMENT.*

Treatment	Turnover Number+ (min^{-1}) 12 hours	48 hours
Saline (n=5)	4182	3418
Thiourea (n=5)	6391	5321

* - turnover number = $\frac{\text{mean Na-K-ATPase activity (umole Pi-hr}^{-1}\text{-mg DNA}^{-1})}{\text{mean Na-K-ATPase quantity (ug alpha-1-mg DNA}^{-1})} \times \frac{1}{60 \text{ min-hr}^{-1}} \times \frac{1}{97000 \text{ umole alpha-1-ug alpha-1}^{-1}}$

+ - no standard error is provided as the turnover number calculation is based on the ratio of means of enzyme activity and enzyme quantity, for a given time point, which were evaluated in different groups of animals (see text).

This is further supported by the fact that the quantity of Na-K-ATPase increased approximately 100% over that of the controls both 12 and 48 hours after thiourea treatment, whereas lung Na-K-ATPase activity increased approximately 200% at 12 hours and 100% at 48 hours (figure 4.5). The turnover number calculations must be interpreted with caution as no estimate of variability can be derived to determine whether the differences between the thiourea and saline groups were statistically significant. Thus, the results of this study would suggest that the observed increase in lung Na-K-ATPase activity may be due to a combined effect of an increased number of enzyme units and an increase in molecular activity.

The results of this study compliment those of Nici et al (94) where an apparent increase in the amount of lung Na-K-ATPase protein was observed during recovery from hyperoxic lung injury in rats. Unfortunately, in the study by Nici et al, the amount of enzyme was not quantified, nor were there attempts to normalize the amount of enzyme to anything but the quantity of protein loaded on their gels. This may be misleading, as hyperoxic injury results in significant influx of protein into the lung (200,201). Thus, lung protein content would differ between the experimental and control animals and would therefore not be a valid variable for normalization of enzyme quantity. As well, there were no corresponding measurements of lung Na-K-ATPase activity,

creating uncertainty as to whether the apparent increase in Na-K-ATPase enzyme quantity would translate into an increase in functional enzyme activity. The present study attempts to account for the above discrepancies by utilizing quantitative immunoblot analysis to demonstrate an increase in lung Na-K-ATPase enzyme concentration during edema resolution, which is normalized to DNA, the amount of which does not change during the recovery from this model of lung injury (figure 4.6, table 7.2). Although the immunoblot procedure cannot distinguish between functional and non-functional Na-K-ATPase enzyme, the fact that lung Na-K-ATPase activity increased at the same time points at which Na-K-ATPase protein concentration increased (figure 4.5) would suggest that the newly synthesized Na-K-ATPase is functional.

As with the studies of Na-K-ATPase activity in lung supernatant preparation, localization within the lung of the changes in Na-K-ATPase content is difficult with the methodology employed. As the immunoblot procedure used in this study specifically detected the alpha-1 subunit of Na-K-ATPase, cell types known to contain predominantly the alpha-2 isoform, such as muscle (202) or adipocytes (55), would not contribute to the observed changes in lung Na-K-ATPase protein content. Bronchoalveolar lavage cells are not likely to contribute these changes either as there were no significant changes in Na-K-ATPase activity in these

cells after thiourea treatment, at least at the 12-hour time point (see chapter 6). Given the methodological difficulties with cellular isolation from lung tissue, in particular with the isolation of AIIC from injured lungs (see chapter 1), and the lack of a reliable technique to isolate alveolar type I cells, further cellular localization of changes in Na-K-ATPase protein becomes difficult. Nici et al (94) qualitatively demonstrated increased immunostainable Na-K-ATPase alpha-1 subunit in rat lung after hyperoxic injury, which localized principally to the alveoli, and particularly to cells resembling AIIC. Although it is tempting to speculate that the increase in lung Na-K-ATPase content observed in this study after thiourea treatment similarly arises from the alveolar region, this speculation must await confirmation by immunohistochemistry or improved lung cell isolation techniques.

In this study, Na-K-ATPase alpha subunit was evaluated by adapting the western blotting technique with immunodetection for quantitative analysis by including on each blot internal standards of known quantity of alpha subunit. A similar technique has been used previously to quantify protein kinase C in lung (90), as well as to quantify Na-K-ATPase alpha-1 and alpha-2 subunits in rat aorta (203) and in rat myocardial and skeletal muscle (204). The method allowed for complete transfer of proteins <200

KDa from gel to membrane, as evidenced by a lack of stainable protein on the post-transblot gel, and for good retention of the Na-K-ATPase alpha subunit protein by the membrane, as evidenced by the lack of immunostainable Na-K-ATPase on adjacent membranes. The McK1 monoclonal antibody proved to sensitively, specifically, and reproducibly detect lung Na-K-ATPase alpha-1 subunit. Internal standards were included on each blot to control for differences between experiments in developing time, alkaline phosphatase activity of the secondary antibody, and minor differences in developing reagent concentrations.

The only other method presently available for quantification of Na-K-ATPase protein involves incubation of samples with tritiated ouabain with quantitation by scintillation counting (111). Although this technique allows for the direct determination of the number of enzyme units in a sample, its application to the lung samples used in this study would be difficult as the alpha-1 subunit of Na-K-ATPase, the predominant isoform in lung (62,63), has the lowest ouabain affinity of all the alpha subunit isoforms (60). Further, the relatively low Na-K-ATPase content of the lung samples analyzed in this study would likely result in relatively high background binding of ouabain, hindering the specific detection of Na-K-ATPase. Finally, as ouabain does not specifically bind to the alpha-1 subunit of Na-K-ATPase, the tritiated ouabain technique

would detect other Na-K-ATPase alpha subunit isoforms present in non-alveolar tissues (eg. muscle (202), adipocytes (55), or macrophages (205)).

The changes in lung Na-K-ATPase protein expression observed in this study could be confirmed by evaluation of lung Na-K-ATPase mRNA expression. The interpritation of changes in tissue mRNA expression, however, is hampered by the need to make the assumption that newly synthesized mRNA is successfully translated into protein. This assumption may not be valid with Na-K-ATPase as a discordant relationship between Na-K-ATPase mRNA transcription and subsequent Na-K-ATPase protein translation has been previously demonstrated (68,85).

The regulatory factors mediating the observed increase in lung Na-K-ATPase content during edema resolution remain speculative. Several of these have been previously discussed (see chapter 1 - section 2b - regulation of Na-K-ATPase activity). With regards to regulation of Na-K-ATPase protein expression within the lung, glucocorticoids, which are found in increased circulating quantities in many critically ill patients (98), have been demonstrated to increase Na-K-ATPase enzyme synthesis in several experimental models (68,84), including whole lung (206) and isolated AIIC (207). The release of anti-diuretic hormone is stimulated by hypoxia (96), a common complication of lung injury, and has also been demonstrated to increase Na-K-

ATPase enzyme synthesis (68,84). Finally, aldosterone is found in increased circulating levels during hypovolemia (98), also a common occurrence with lung injury, and is well known to stimulate Na-K-ATPase synthesis in kidney (68). Early evidence would suggest aldosterone can also increase Na-K-ATPase expression in rat AIIC as well (191). The role these potential regulatory factors play in mediating changes in lung Na-K-ATPase activity and the potential use of these factors as therapeutic agents for enhancing lung sodium and liquid clearance are important topics for future study.

In summary, the quantity of Na-K-ATPase protein in lung is increased during the resolution from high permeability pulmonary edema at times when lung Na-K-ATPase activity is also increased. This suggests that one mechanism by which lung Na-K-ATPase activity increases is by new Na-K-ATPase enzyme synthesis. There is suggestive but inconclusive evidence that increases in the molecular activity of Na-K-ATPase may also contribute to changes in lung Na-K-ATPase activity.

CHAPTER 8

SUMMARY AND FUTURE DIRECTION

8.1 SUMMARY

As opposed to the relatively large amount of data acquired concerning the methods and mechanisms of lung injury and the subsequent induction of pulmonary edema, relatively little information has been collected concerning the resolution from this state. The ultimate goals in clinically oriented research with respect to pulmonary edema are more rational and effective approaches to patient care. The initial goal of laboratory-oriented research concerning the resolution of pulmonary edema is a better understanding of the cellular and tissue mechanisms underlying this process.

Based on experiments performed on AIIC monolayers and isolated perfused animal lungs, an initial understanding of the process of edema resolution was obtained. Lung liquid clearance was found to be dependent on the active transport of sodium by the alveolar-capillary barrier. The sodium dependence of this process has been confirmed in animal models of experimentally induced alveolar flooding (18,19). A single study has confirmed the active nature of lung liquid clearance in humans (5). Active sodium transport had not been adequately studied, however, during the recovery from realistic *in vivo* models of pulmonary edema. Given the controlled nature of *in vitro* and *ex vivo* isolated lung

studies (lack of local and systemic hormonal influences...etc), confirmatory in vivo studies were indicated. Further, given that experimentally induced alveolar flooding is a relatively unrealistic simulation of the human condition of pulmonary edema, these in vivo confirmatory studies should utilize more realistic models of lung edema. The overall goal of these studies was to demonstrate the stimulation of active sodium transport during the resolution from an in vivo model of thiourea-induced pulmonary edema. As transepithelial sodium fluxes could not be evaluated in vivo, another marker of active sodium transport was required. Given that the Na-K-ATPase enzyme is the only known mechanism for the basolateral extrusion of sodium once absorbed from the alveolar surface, it was reasonable to monitor the activity of this enzyme as an indirect indice of active sodium transport. The thiourea model of lung injury, although having no direct clinical counterpart, was utilized as it is a well defined model of pulmonary edema that has several features in common with human forms of lung injury (eg. sepsis). In particular, thiourea induces characteristic histological changes in the endothelial cells of the alveolar region (149-151) and results in high-permeability pulmonary edema (149,152,153).

The first task in these studies was to validate the techniques for the preparation of lung tissue and for the measurement of Na-K-ATPase activity in such tissue.

Although the general methodology for the measurement of Na-K-ATPase activity had been previously characterized, as this was a new application of the technique to lung tissue, it was important to verify optimal assay conditions. As outlined in chapter 3, the lung preparation procedure provided for good recovery of protein, DNA, and Na-K-ATPase, and the Na-K-ATPase assay provided for the reliable evaluation of Na-K-ATPase activity under optimal conditions.

With the Na-K-ATPase assay methodology validated, one could proceed with the evaluation of lung Na-K-ATPase during the resolution from lung edema. In chapter 4 it was demonstrated that lung Na-K-ATPase was activated during the recovery from thiourea-induced pulmonary edema. The time course of Na-K-ATPase activation in relation to the recovery of lung edema suggested that active sodium transport was occurring during this process. Several questions arose based on these findings.

The first question concerned possible direct or indirect effects of thiourea on the Na-K-ATPase enzyme that may have accounted for the observed changes in lung Na-K-ATPase activity. In chapter 5 it was shown, firstly, that thiourea had no direct effects on lung Na-K-ATPase in vitro. Secondly, alveolar flooding, in the absence of thiourea, activated Na-K-ATPase to a similar degree as was observed during the recovery from thiourea-induced edema. Together, this suggested that it was the presence of edema, or the

physiologic consequences of edema, that was mediating the changes in lung Na-K-ATPase activity during edema recovery.

The second question that arose from the findings of chapter 4 concerned the possible contribution of bronchoalveolar lavage cells to the observed increase in total lung Na-K-ATPase activity during edema recovery. In chapter 6, it was shown that changes in bronchoalveolar cells could not account for this increase.

A final question concerned the general mechanisms underlying the observed increase in lung Na-K-ATPase activity. In chapter 7 it was demonstrated that the amount of lung Na-K-ATPase enzyme increased during the recovery from pulmonary edema. This suggested that one mechanism by which lung Na-K-ATPase activity is modulated is by a change in the number of enzyme units.

In summary, these studies have shown that lung Na-K-ATPase is activated during the resolution from a realistic in vivo model of high permeability pulmonary edema. This suggests that active sodium transport is operative during this process, as has been suggested by several ex vivo and in vivo studies of alveolar flooding (9,18,19,21). Thus, in the progression of research into the mechanisms underlying the resolution of pulmonary edema, these studies have provided confirmatory evidence for a model of edema resolution that involves active sodium transport using a realistic in vivo model of lung injury. This is one

important and necessary link in the very long chain which connects the laboratory bench with the hospital bed.

8.2 FUTURE DIRECTION

Consistent with most scientific studies, this study raises more questions than it answers.

A recurring uncertainty with these studies is the difficulty in localizing within the lung where the observed changes in Na-K-ATPase activity are occurring. One approach often taken to address this concern involves the differential cell isolation from the tissue of interest followed by measurement of enzyme activities in these isolated cells. Unfortunately, this approach is not readily applicable to the lung as the only accessible cell types are alveolar type II and bronchoalveolar lavage (BAL) cells. This study addresses Na-K-ATPase activity changes in BAL cells. An analysis of AIIC would be limited by difficulties with their isolation from injured lungs and their questionable functional status once in culture (see chapter 1). Given that the quantity of Na-K-ATPase also increases during the resolution from high-permeability pulmonary edema, an alternative approach for the localization of changes in Na-K-ATPase quantity can be envisioned. This would involve utilizing specific anti-Na-K-ATPase antibody for immunohistochemical staining of fixed lung specimens (94). The evaluation would be qualitative and would involve comparison of the amount of bound antibody between control

and experimental lungs at times during the recovery from lung injury. A similar approach could utilize radiolabeled cDNA probes specific for Na-K-ATPase mRNA (208). While this approach would address changes in the quantity of lung Na-K-ATPase, it would not localize changes in Na-K-ATPase molecular activity.

A second area meriting further investigation concerns the relationship between arterial pH and PCO_2 and lung Na-K-ATPase activity during the recovery from alveolar flooding (see chapter 5). The next step would be to determine whether changes in pH and PCO_2 also influence the rate of lung liquid clearance in addition to Na-K-ATPase activity. Given the limited variability in lung liquid clearance rates observed in the experiments outlined in chapter 5, a relatively large number of animals would need to be studied, with pH and PCO_2 levels varying over a wide range. A better understanding of the relationship between these variables and lung sodium and liquid transport has clinical relevance. A respiratory and/or metabolic acidosis commonly accompanies pulmonary edema and is often corrected either with systemic alkali or by increases in ventilation. Although severe acidemia may have deleterious hemodynamic effects (209), a mild acidosis (eg. pH 7.25-7.35) may have beneficial effects on lung sodium and liquid transport.

Finally, and most important with regards to clinical application, is investigation into the factors modulating

lung sodium and liquid clearance. While many agents have been evaluated in vitro, relatively few have been studied in vivo. Those evaluated in vivo include aldosterone, beta agonists, and glucocorticoids.

Aldosterone is known to activate Na-K-ATPase and net sodium transport in kidney (68) and lung (81,82) and has recently been demonstrated to increase lung liquid clearance in isolated perfused rat lungs (83). In the latter study, aldosterone was administered by aerosolization, which is an attractive approach for drug delivery to the lungs in that maximum alveolar delivery is achieved with minimal systemic side-effects. The thiourea-induced lung injury model used in these studies would be a suitable model for study into the effects of aldosterone on the recovery from an in vivo model of pulmonary edema. One could compare the time course of recovery of wet/dry ratios after induction of edema with thiourea between animals treated with aldosterone versus untreated controls. Lung Na-K-ATPase activity could be evaluated as an index of active sodium transport. As there is ample clinical experience with mineralocorticoid therapy in humans with adrenal insufficiency (210), the transition from animal experimentation to therapy for human disease would likely be easier when compared to implementation of novel therapeutic agents.

Beta-agonsists are known to a activate Na-K-ATPase in AIIC monolayers (24) and increase net sodium transport in

isolated perfused lungs (20). In an alveolar flooding model in sheep, administration of the beta agonist terbutaline, either intravenously or intra-alveolarly, increased lung liquid clearance (18). It is conceivable that beta agonists, already in common use for the therapy of bronchospasm, when administered in the same manner to humans may have beneficial effects on edema resolution. Glucocorticoids have also been demonstrated to activate Na-K-ATPase in kidney (68,84) as well as increase lung Na-K-ATPase protein expression in fetal rats (206). Glucocorticoids have been subjected to several randomized, placebo-controlled clinical trials for the therapy of ARDS or septic patients at risk of ARDS (211-213). Unfortunately, no clinical benefits were realized in any of these trials (211-213). Perhaps the most important lesson to be learned from these trials is that agents of suspected clinical efficacy for the treatment of pulmonary edema can and should be subjected to such randomized trials prior to clinical acceptance.

The present therapy for pulmonary edema is primarily supportive in nature, with virtually no therapy actually accelerating lung liquid clearance. An understanding of the mechanisms and regulation of the edema resolution process, and ways to potentially accelerate this process in animal models will hopefully lead to more useful and rational

approaches to therapy for the human condition of pulmonary edema - a common and potentially fatal clinical entity.

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