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CONTROL OF LUNG LIQUID THROUGHOUT LATE GESTATION AND LABOUR

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SUMMARY

1. The fetal lung is filled with a large volume of liquid that must be cleared to allow gas exchange after delivery. Whether lung liquid clearance begins prior to labour, or occurs entirely within labour is in dispute. Furthermore, the mechanisms that initiate lung liquid clearance before delivery have not been fully elucidated. Yet, these mechanisms ensure that the alveolar space is liquid free in postnatal life and their understanding is essential to prevent and treat a large proportion of the common respiratory diseases in the newborn.

2. We suspected that the essential difference between studies reporting a pre-labour decline in lung liquid volume and those reporting a pre-labour increase, followed by within-labour initiation of lung liquid clearance, could relate to the different volume tracers used; radio-iodinated serum albumin (RISA) was used in the former case and Blue Dextran (BD) in the latter.

3. We tested the reliability of both tracers in fetal sheep at 124 and 142 days gestation (G124 and G142; term = G147). BD was found to contravene two critical assumptions of the indicator dilution technique. First, BD bound to the pulmonary epithelium, with greater binding at G142 than at G124; such binding must lead to an age-dependent overestimate of lung liquid volume. Second, the process of mixing, an integral part of the indicator dilution technique, caused a large increase in optical background absorbance, equivalent to an increase in BD concentration. This effect would predispose to an underestimate of lung liquid volume. Together these sources of error render BD an unsound volume tracer. By contrast, very little RISA bound to the

epithelium or crossed into the fetal circulation. Thus RISA provides reliable estimates of lung liquid volume in fetal sheep.

4. We again addressed the question of a pre-labour decline in lung liquid volume (V_L) by making longitudinal determinations of V_L and secretion rate (J_v) over the last week of gestation. V_L declined in two phases, the first beginning 70 hours before the fetus was in advanced labour, the second beginning within labour. J_v also declined in two phases, but reabsorption of liquid was not observed except in a single fetus in advanced labour.

5. To examine whether ventilation-associated stretch of the lung could trigger trans-epithelial liquid clearance, we cyclically expanded the lung in late gestation fetuses with artifical lung liquid. Lung liquid secretion rate fell in most fetuses and absorption of liquid occurred in some animals. The effect was not reversed by amiloride blockade of active Na^+ transport, suggesting that lung stretch acts through an increase in epithelial permeability rather than via activation of Na^+ channels.

6. In conclusion, clearance of lung liquid begins well before commencement of labour in the fetal sheep, with labour being associated with an acceleration of clearance. Further, substantial clearance of lung liquid occurs before any reabsorption of liquid across the pulmonary epithelium is observed.

7. These findings highlight the importance of natural labour for normal lung adaptation at birth and suggest that the well-documented epidemiological finding of greater respiratory morbidity after elective caesarean delivery results from failure of the fetus to experience a large decline in lung liquid volume before birth.

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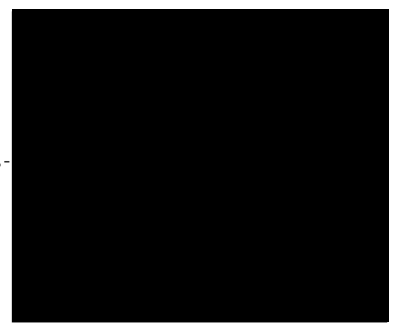
DECLARATION OF AUTHENTICITY

I declare that, with the exception of the afore-mentioned acknowledgments, the ideas, implementation of experiments, the data analysis and the interpretation of the findings are the work of Riccardo E. Pfister.

This thesis contains no material which has been submitted for the award of any other degree or diploma in any university or other institute, and contains no material previously published or written by another person, except where due reference is made in the text.



DEDICATION

This dedication with my deep feelings goes to my family, my mother Erennia and my father Peter who have grown me and supported me all along. It goes to my brothers and sisters scattered over the globe who have interacted with me and comforted me by their universal presence. It goes also to my larger family, which has always been close to my heart despite the distance separating my home country Switzerland from Australia.

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ABBREVIATIONS

ABBREVIATION	MEANING
AVP	arginine vasopressin
ATP	adenosine triphosphate
BD	blue dextran
cAMP	adenosine 3',5'-cyclic monophosphate
CF	cystic fibrosis
EMG_d	electromyogram of diaphragm
ENaC	amiloride-sensitive epithelial sodium channel
GA	gestational age
J_v	lung liquid secretion rate
LL	lung liquid
NO	nitric oxide
P_{AF}	amniotic fluid pressure
RDS	respiratory distress syndrome
RISA	radio iodinated serum albumin (I^{125} if not stated otherwise)
TRH	thyrotropin-releasing hormone
V_L	lung liquid volume
RV	residual volume
FRC	functional residual capacity
TLC	total lung capacity
PEEP	positive end expiratory pressure
CPAP	continuous positive airway pressure

CHAPTER ONE

GENERAL INTRODUCTION

GENERAL INTRODUCTION

Survival at birth is dependent upon an immediate transition from placental to pulmonary gas exchange in the newborn. Adequate development of the pulmonary parenchyma in which gas exchange occurs is a clear prerequisite for postnatal survival, with the establishment of a large alveolar surface area, the elaboration of a thin but extremely elastic and tough diffusion membrane, together with a matching vascular network being obvious examples. This aspect of respiratory development has been reviewed extensively (Schmidt, 1966; Levine *et al*, 1970; Hasleton, 1972; Wiebe and Laursen, 1995; Massaro and Massaro, 1996; West and Mathieu-Costello, 1999), and will not be addressed in this Thesis. An additional prerequisite for postnatal survival is the development of the surfactant system of the lung, which has the role of reducing surface tension at the air-liquid interface on the luminal surface of the alveoli. This is achieved by the functional maturation of the Type II cells of the lung of which one chief role is to produce and secrete a complex mixture of phospholipids and proteins which spread over the alveolar lining and reduce surface tension. This aspect of development has been extensively studied and reviewed (Hills, 1987; Jobe, 1993; Goerke, 1998, Hills and Masters, 1998; Jobe and Ikegami, 1998; Griese, 1999) and also lies outside the scope of this Thesis. What this Thesis focuses on is the transformation of the lung from an organ filled with liquid during fetal life to one that is in essence almost entirely dry in postnatal life. This transformation occurs in the perinatal period and ensures that arterial oxygen levels in the newborn rise rapidly from the low levels characteristic of fetal life to the high levels typical of the healthy newborn and adult. At the time this Thesis was started it was clear from epidemiological studies that considerable lung pathology in the newborn was associated with persistence of an excess of liquid in the lung. However, the time course with which

liquid was cleared from the lung had not been established, nor had the mechanisms by which liquid clearance is achieved been completely elucidated.

1.1 The Fetal Lung

There is abundant evidence that the lung of mammalian fetuses is not collapsed, but filled with liquid to a volume that approximates postnatal functional residual capacity (30 ml.kg^{-1}). It is also clear that the source of lung liquid is the pulmonary epithelium itself, which for most of gestation secretes liquid into the future airspace. These points will be addressed in detail in Sections 2.2.2 and 2.7. Secretion of lung liquid from the pulmonary epithelium is an active, energy consuming process, based on the movement of Cl^- ions across the luminal surface of the epithelium (see Section 2.4) which results in liquid movement into the future airspace; in fetal sheep secretion occurs at a rate of 3-4 ml.kg^{-1}.hr^{-1}. The rate of liquid movement approximates fetal urine output and it vastly exceeds that required to distend the growing lung to its normal volume. It can be estimated that more than 99% of the liquid secreted by the lung flows out of the fetus via the future airways, becoming, along with fetal urine, the main source of amniotic fluid.

In contrast to the postnatal condition, the lung has no known function that is vital to the fetus. However, appropriate growth and development of the pulmonary parenchyma during fetal life is essential for the survival of the newborn, and these processes are critically affected by the volume of liquid in the lung and in the amniotic sac. By contributing both to lung liquid and to amniotic fluid volumes, the secretory function of the pulmonary epithelium therefore plays a crucial role in lung development. This view is supported by evidence showing that lung growth and development are dramatically affected by alteration to the volume of liquid in the lung or in the space surrounding the fetus, resulting

either from naturally occurring fetal pathologies or from experimental manipulations as detailed later in Section 2.9.

1.2 The Post-Natal Lung

The alveolar surface of the newborn and adult lung needs to be virtually "dry" to facilitate gas exchange. In the classic model of lung function, a small volume of liquid remains in the lung, creating a very thin layer of liquid that lines the alveolar surface. It is envisaged that surfactant spreads across this "alveolar lining layer" covering the entire surface of the alveoli, thereby reducing surface tension at the air-liquid interface. The film of residual lung liquid might also play a role as a vector for debris which is cleared via the action of macrophages in the alveolus (Pavia, 1991) and then by the muco-ciliary epithelium of the airways (Robertson *et al*, 1976). In order to maintain the lung in its "dry" state, considerable evidence now shows that any excess liquid in the alveoli is actively removed by the action of Na^+/K^+-ATPase pump and specific Na^+ channels on the luminal surface of the pulmonary epithelium. The pathways of cellular Na^+ absorption have been established in considerable detail, and it is known that malfunction of the Na^+ transport mechanism gives rise to respiratory failure and death soon after delivery. Active Na^+ transport will be addressed extensively in Sections 2.5, 2.6 and 2.10.

1.3 Adaptation Of The Perinatal Lung

The essential change that occurs in the lung at birth is that the very large volume of liquid that distends the late-gestation fetal lung is cleared until all that is left is the small volume of liquid making up the alveolar lining layer. When the process of lung liquid clearance begins is a key question that has not yet been resolved (see Section 2.10), although it appears likely from epidemiological work that labour plays an important role in the

process. However, whether a substantial volume of liquid remains in the lung at the time the fetus is delivered was not known when this program began. A further issue that needed resolution is the mechanism by which clearance is achieved. One possibility is that the Na^+ pump is the chief mechanism involved. However, its capacity to remove the vast volume of liquid in the lung of the late-gestation fetus could be questioned, especially since available evidence suggested that the pump is activated only in the final 1 – 2 hours of labour. The motivation for carrying out the studies that comprise this Thesis is that resolution of these arguments would improve our understanding of perinatal lung adaptation, and its frequent pathologies, and might point further research towards new treatment strategies.

CHAPTER TWO

LITERATURE REVIEW

LITERATURE REVIEW

A large body of research has been directed at understanding the physiological mechanisms that give rise to the normal growth and development of the fetal lung and the manner by which the liquid-filled fetal lung becomes adapted to its postnatal function. These issues represent the focus of the following review as well as the focus of the research comprising this Thesis. As one of the most amazing events in the adaptation to air-breathing at birth is the diametrical transformation of the lung from being liquid-filled to air-filled, the approach taken here is to focus attention on lung liquid, both from the perspective of the mechanisms underlying its production and the manner in which its volume is controlled throughout fetal life and perinatally. As a first step, it is necessary to assess methods that have been used to study fetal lung liquid.

2.1 Lung Liquid Measurements

A number of techniques have been used to determine lung liquid volume and secretion rate, together with the impact on these two variables of a variety of experimental manipulations, as well as the onset of key developmental periods such as labour. The largest number of publications report on fetal sheep, but other species have also been used, including rodents, guinea-pigs, rabbits, rats and mice, and more rarely goats, dogs, rhesus monkeys, ferrets and marine turtles (see Table 2-1). A variety of methods for examining lung liquid dynamics has been used, including the wet/dry weight ratio of the lung, a technique that allows a single estimate to be made of lung liquid volume (Bland *et al*, 1979) and direct measurements of flow rates (Setnikar *et al*, 1959; Adams *et al*, 1963a; Enhörning and Adams, 1965; Maloney *et al*, 1975) during drainage or instillation of liquid (Alcorn *et al*, 1977; Moessinger *et al*, 1990). These techniques however do not lend themselves to longitudinal or long-term studies in vivo, a requirement if we are to

understand the control of lung liquid over a long span of gestation. The vast majority of studies therefore utilise impermeant tracer techniques, which allow simultaneous estimates of lung liquid volume and secretion rate via dilution of a tracer. These techniques have effectively allowed volume and secretion rate determinations to be repeated over periods of many weeks in the fetal sheep, thereby facilitating the study of developmental changes in the control of lung liquid.

Table 2-1. *Selection of references on different species used in lung liquid studies*

Human	Potter, 1941; O'Brodovich, 1996; Barker *et al*, 1997; Smith *et al*, 2000
Rhesus monkey, macaque	Prueitt *et al*, 1979; O'Brodovich, 1992
Sheep	Reynolds, 1953; Adams *et al*, 1963; Adams *et al*, 1963; Boston *et al*, 1965; Enhörning, 1965; Humphreys *et al*, 1967; Boston *et al*, 1968; Adamson *et al*, 1969; Normand *et al*, 1971; Adamson *et al*, 1973; Walker, 1973; Adamson *et al*, 1975; Egan *et al*, 1975; Maloney *et al*, 1975; Scarpelli *et al*, 1975; Alcorn *et al*, 1977; Kitterman *et al*, 1979; Alcorn *et al*, 1980; Bland *et al*, 1982; Walters *et al*, 1982; Egan *et al*, 1984; Harding *et al*, 1984; Murai *et al*, 1984; Perks *et al*, 1985a; Cassin *et al*, 1986; Harding *et al*, 1986; Dickson *et al*, 1987; Dickson *et al*, 1987; Barker *et al*, 1988; Hooper *et al*, 1988; Walker *et al*, 1988; Berthiaume *et al*, 1989; Dickson *et al*, 1989; Barker *et al*, 1990; Barker *et al*, 1990; Wallace *et al*, 1990; Walters *et al*, 1990; Barker *et al*, 1991; Dickson *et al*, 1991; Carlton *et al*, 1992; Davis *et al*, 1992; Campos *et al*, 1993; Cassin *et al*, 1994; Chapman *et al*, 1994; Wlodek *et al*, 1994; Hooper *et al*, 1995; Nardo *et al*, 1995; Berger *et al*, 1996; Hooper *et al*, 1996; Wallace *et al*, 1996b; Cummings, 1997; Lines *et al*, 1997; Berger *et al*, 1998; Nardo *et al*, 1998; Pfister *et al*, 1999; Berger *et al*, 2000
Goat	Cassin *et al*, 1982; Perks *et al*, 1985b
Dog	Agostoni, 1959; Conhaim *et al*, 1983; Berthiaume *et al*, 1988
Guinea pig	Garrad-Nelson *et al*, 1990; Perks *et al*, 1990; Thom *et al*, 1990; O'Brodovich *et al*, 1992; Kindler *et al*, 1993; Perks *et al*, 1993; Garrad-Nelson *et al*, 1996; Woods *et al*, 1996
Rabbit	Jost *et al*, 1948; Wyszogrodski *et al*, 1974; Enhörning *et al*, 1977; Bland *et al*, 1979; Bergman *et al*, 1980; Kanjanapone *et al*, 1980; Vejlstrup *et al*, 1994
Rat	Basset *et al*, 1987b; Garat *et al*, 1995; Yasui *et al*, 1997
Mouse	Hummler *et al*, 1996
Ferret	Webber *et al*, 1989
Marine turtle	Maloney *et al*, 1989

2.1.1 Impermeant tracer

The indicator dilution technique requires a precisely measured amount of impermeant tracer to completely mix and dilute in the lung liquid to be measured. Volume is then determined by dividing the quantity of tracer added by its concentration measured in a small sample of lung liquid. As secretion reduces tracer concentration with time, and absorption increases it, repeated determinations of the tracer concentration allow calculation of secretion or absorption rate.

Table 2-2. *Key assumptions of the indicator dilution technique*

Assumption	**In relation to lung liquid determination**
evenly distributed	even distribution, small variability
stable	no trapping by mucus or pulmonary epithelium
	no degradation or precipitation
impermeant	no passage across the epithelium
inert	no alteration of liquid secretion/absorption rate
	no interference with simultaneously used drugs or diluents
practical	simple and accurate determination of concentration
	accurate determination in opaque (surfactant-containing) liquid
	simultaneous use with other indicators

The key assumptions of the indicator dilution technique (see Table 2-2) upon which its accuracy relies, are 1) that the tracer must be evenly distributed throughout the liquid whose volume is being measured, 2) the tracer must not react with the cells lining the chamber containing the liquid being measured, 3) the properties of the tracer being relied upon for measurement of its concentration must not change during the experiment, 4) the tracer must remain within the compartment under study and 5) it must not alter biological processes that could affect the mechanisms that control the secretion or volume of the liquid under study (Moore *et al*, 1969; Scarpelli *et al*, 1975). The earliest method for

making determinations of lung liquid volume and secretion rate in the fetal sheep involved the use of radio-labelled inulin (Normand *et al*, 1971), but this was soon replaced by radio-labelled albumin (Olver and Strang, 1974; Scarpelli *et al*, 1975) and still later by the large molecule blue dextran.

Radio labelled serum albumin (RISA)

Radio-labelled serum albumin had the advantage of remaining confined to the lung compartment due to its large size (Egan, 1976) without being a foreign substance that might interfere with physiological processes, since albumin is the principal naturally occurring protein of lung liquid. I^{131} was bound to serum albumin to facilitate determination of tracer concentration. RISA satisfied all the important characteristics required of a dilution indicator, i.e. RISA manifested good stability, with only minimal shift of I^{131} to other proteins, and there was no tracer loss across the pulmonary epithelium into the blood for at least the first 2 to 2 1/2 hours after introduction into the lung compartment (Scarpelli *et al*, 1975). Furthermore, because of the low concentration of protein required, possible functional interference with lung liquid turnover by oncotic pressure changes resulting from added albumin was calculated to be negligible. Finally, although loss of tracer through binding with the lung epithelium was not excluded in the validation study of Scarpelli performed on fetal sheep, the binding of large protein molecules to the alveolar membrane was considered unlikely, or to be likely to occur only very slowly. Failure to attempt to assess the degree of possible binding of albumin to the pulmonary epithelium remains, however, a weakness of the validation of the technique.

Blue Dextran

Some authors (Cassin and Perks, 1982; Perks and Cassin, 1982; Hooper *et al*, 1988)

abandoned the use of RISA in favour of the high molecular weight dye Blue Dextran (BD). Dilution of this dye could be readily and accurately measured by spectrophotometry, making quantitative determinations as convenient as with RISA. Although there have now been many studies involving Blue Dextran, there has been only one validation of the method published for lung liquid measurements. This study reported that lung liquid volumes and secretion rates determined with BD were the same as those derived from RISA (Beierle *et al*, 1996), suggesting that Blue Dextran is as reliable as RISA for determining lung liquid volume and secretion. This report, however, does not provide a full validation of the BD method, since there was no rigorous evaluation of whether BD satisfies the crucial assumptions of the indicator dilution technique (see Table 2-2) when used in the fetal lung.

2.2 Lung Liquid: Composition And Production

Until 1913, when Addison (Addison and Howe, 1913) reported that the fetal lung was distended and liquid filled, it was generally believed that the lung was empty and collapsed, ready to open through the action of elastic recoil at birth. Since Addison's discovery, an abundance of work has confirmed the ubiquity of liquid in the lung of fetal mammals: human (Potter and Bohlender, 1941), rabbit (Jost and Policard, 1948), sheep (Reynolds, 1953; Adams *et al*, 1963a; Boston *et al*, 1965), dog (Agostoni, 1959), macaque (Prueitt *et al*, 1979), goat (Cassin and Perks, 1982), rat (Basset *et al*, 1987b), guinea pig (O'Brodovich and Merrit, 1992), rhesus monkey (O'Brodovich and Merrit, 1992) and mouse (Hummler *et al*, 1996). No investigation has been undertaken to determine the volume and secretion rate of lung liquid of marsupial mammals, perhaps because of their small size when they leave the uterus and attach to the teat. Limited evidence of the presence of lung liquid has been presented for at least three oviparous vertebrate embryos,

the chick, an Australian reptile, the bearded dragon *Pagona vitticeps* (Johnston *et al*, 2000), and the marine turtle (Maloney *et al*, 1989).

2.2.1 Lung liquid composition

Lung liquid has long been known to have a distinctly different composition from that of amniotic fluid (Adams *et al*, 1963b). In subsequent work, the composition of lung liquid has been compared to other body fluids on a number of occasions (Boston *et al*, 1968; Adamson *et al*, 1969; Mescher *et al*, 1975; Strang, 1977), as summarised in Table 2-3.

Table 2-3. *Composition of lung liquid compared to other body fluids*

	lung liquid	**lung lymph**	**amniotic fluid**	**plasma**
[Na^+]	150 (1.3)	147 (0.7)	113 (6.5)	150 (0.7)
[K^+]	6.3 (0.7)	4.8 (0.5)	7.6 (0.8)	4.8 (0.2)
[Cl^-]	157 (4.1)	107 (0.9)	87 (5.0)	107 (0.9)
[HCO_3^-]	2.8 (0.3)	25 (0.8)	19 (3.0)	24 (1.2)
[phosphates]	<0.02		3.2	2.3
[urea]	7.9 (2.7)		10.5 (2.4)	8.2 (1.4)
pH	6.27	7.31	7.02	7.34
[protein] $g.l^{-1}$	< 10	142 (108-179)	<10	220 (177-270)
protein oncotic pressure (cm H_20)	0.027	2.36	0.1	3.46

Mean (SE) in $mmol.l^{-1}$, unless otherwise indicated (from Boston et al, 1968; Adamson et al, 1969; Strang, 1977; Cotton et al, 1988; O'Brodovich and Merrit, 1992).

The main point of difference to amniotic fluid is its almost 50% larger Cl^- concentration, underlining the essential role of Cl^- in lung liquid production. A further difference, the low HCO_3^- concentration in lung liquid, raises the possibility of bicarbonate absorption from

the lung lumen, for which there is some evidence in the fetal (Shaw *et al*, 1990) and adult lung (Nord *et al*, 1987). The Na^+ content is also clearly higher in lung liquid than in amniotic fluid, but similar to plasma and lung lymph. Another typical characteristic of lung liquid is its very low protein concentration and protein oncotic pressure, similar to amniotic fluid values and far less than in plasma. It could be speculated that this gradient is of importance for water transport from the lung to the vascular compartment in the perinatal period.

Some characteristic maturational changes in ion concentrations are now known to occur during gestation (Olver *et al*, 1981). Whilst no data are available for the first half of gestation, during the second half lung liquid concentrations of Na^+ remain stable, while Cl^- and K^+ increase in parallel with their increase in plasma with approaching term. Interestingly, the plasma/lung liquid ratio is lower for Cl^- (0.7) than for K^+ (0.95) but it remains almost unchanged throughout gestation (Olver *et al*, 1981). Bicarbonate falls considerably, particularly when compared with the unchanged plasma levels, from around 10 $mmol.l^{-1}$ around mid-gestation to 2 $mmol.l^{-1}$ at term, and proteins remain low despite a substantial rise in plasma levels.

2.2.2 Lung liquid production

Over a century ago, Preyer reported that the guinea-pig fetus could be induced to inhale amniotic fluid by strong stimulation of the vibrissae (Preyer, 1885). This observation may have given rise to the view that the liquid present in the fetal lung was inhaled amniotic fluid (Towers, 1959). However, such a view was rendered untenable by the finding that lung lobes below atretic bronchi were liquid-filled in neonates (Potter and Bohlender, 1941). Thus, it was clear that lung liquid originates distally, i.e. from the lung itself.

Similar observations of grossly liquid-distended lungs were made by chance during the course of an endocrine study in which the whole neck was ligated in the fetal rabbit, completely obstructing the trachea (Jost and Policard, 1948). On the basis of this finding, it was suggested that the fluid present in fetal rabbit lungs was not amniotic fluid, but an active secretion of the pulmonary epithelium (Jost and Policard, 1948). The fact that lung liquid was later shown to differ in composition from amniotic fluid (Adams *et al*, 1963b; Boston *et al*, 1968; Adamson *et al*, 1969; Mescher *et al*, 1975; Strang, 1977) provided further evidence that it could not be amniotic fluid. Studies by a number of groups (Adams *et al*, 1963b; Nelson *et al*, 1963; Adamson *et al*, 1969; Scarpelli *et al*, 1975), and particularly the mechanistic studies of Strang and his co-workers (Olver *et al*, 1973; Olver and Strang, 1974; Walters *et al*, 1982), have added greatly to our knowledge of lung liquid and the way it is produced.

Some time after the crude procedure of whole neck ligation had led to the discovery that the lungs must produce liquid (Jost and Policard, 1948), confirmation was obtained in experiments where the trachea was selectively ligated in the intact healthy fetus (Carmel *et al*, 1965). The occurrence of considerable lung distension against a hydrostatic pressure gradient of several cm H_2O (Lanman *et al*, 1971) suggested that the mechanism producing lung liquid might be active. A later study demonstrated the active nature of lung liquid secretion by measuring unidirectional ion fluxes across the fetal sheep lung epithelium (Olver and Strang, 1974). By comparing these results to predicted ion fluxes based on electrochemical gradients (Koefoed-Johnsen and Ussing, 1953), Olver concluded that the mechanism of liquid secretion involves an active movement of halide ions, of which Cl^- appears to play the essential role (see for more details Section 2.3.5).

2.3 Factors Affecting Liquid And Ion Movement Across The Epithelium

Liquid and ions may move in either direction across the lung epithelium; movement in the direction from interstitium to the future airspace constitutes secretion, whereas the reverse movement represents absorption. Water moves across the epithelium in response to a difference in the hydrostatic or osmotic pressure between the lumen and the interstitium. Ions may move passively in response to a difference in concentration or electrical potential across the epithelium, the rate being determined by the permeability of the barrier itself. The final mechanism by which liquid or ions move across the epithelium is via active transport.

2.3.1 Permeability of the lung epithelium

To retain the liquid that occupies the alveolar space, given that its composition differs from interstitial fluid and plasma, the lung epithelium has to be relatively impermeable to small molecules, otherwise a rapid re-balance would occur. The diffusion properties of different molecules and substances across the epithelium in relation to their molecular size have been determined experimentally. These properties have allowed quantification of the permeability of the epithelium in terms of "pore" size (Normand *et al*, 1971). During fetal life, the lung epithelium is very "tight", so that it effectively excludes all molecules larger than sucrose; that is, with an effective radius larger than 0.55 nm (Normand *et al*, 1971) or 0.64-0.66 nm (Olver *et al*, 1981). From early gestation to term, epithelial permeability to small polar non-electrolytes has been shown not to change significantly (Olver *et al*, 1981). However an increased pore size has been demonstrated to result from lung expansion (Egan *et al*, 1975). A peak pore size of 4.00 nm was determined during the first 12 postnatal hours (Egan, 1976), with a subsequent drop to 1.1 nm measured at 12 to 60 hours after birth (see Table 2-4).

Table 2-4. Alveolar membrane pore size in fetal sheep and immediately after birth and 12-60 hours following birth (from Egan, 1976)

	n	Pore radius (nm) average	Range
fetal	14	0.55	0.5-0.6
at birth	7	4.0	1.5-5.6
12-60 h	4	1.1	0.7-1.4

2.3.2 Hydrostatic pressure

Hydrostatic pressure gradients between the alveolar lumen and interstitium exert an influence on trans-epithelial liquid movements. Positive hydrostatic pressures, i.e. forces counterbalancing active secretory forces, might be expected in the completely liquid filled fetal lung. In this context, the relevant hydrostatic pressure is that between the lumen and the interstitial space of the lung. We do not have values for this pressure gradient in the fetus, but it is likely to be similar to that between the tracheal lumen and the intrapleural space, which is reported to be 2.8 – 3.4 cm H_2O in the fetal sheep (Vilos and Liggins, 1982). Since curarisation did not alter the pressure gradient, it was concluded that positive intra-tracheal pressure is generated by continuous lung liquid secretion in the presence of the outflow resistance of the airways (Vilos and Liggins, 1982). It is clear, therefore, that secretion continues in the face of a positive pressure in the trachea. A later study reported that intra-tracheal pressures (compared to P_{AF}) under control conditions ranged from 1.4 - 3.7 cm H_2O, when outflow of liquid from the lung was free to pass through an exteriorised tracheal loop; this pressure rose to 5.8 ± 0.5 cm H_2O when the loop had been obstructed for several days (Nardo *et al*, 1998). Importantly, lung liquid secretion had fallen to zero at an intra-tracheal pressure of 5.7 cm H_2O (Nardo *et al*, 1998), raising the possibility that at pressures approximating this level the secretory force is balanced by hydrostatic pressure. To date, attempts to relate secretion rate to hydrostatic pressure, by altering intra-tracheal

pressures between 2 and –5 cm H_2O, have failed, mainly due to difficulties in quantifying very small changes in pressure and secretion in active animals (Boston *et al*, 1968).

Negative hydrostatic pressures can be expected in the alveolar lining layer of the air-filled lung, resulting from the surface tension generated at the liquid-air interface; this pressure would act to impede liquid reabsorption resulting from Na^+ transport across the pulmonary epithelium. As considered later (Section 2.10.2), it seems likely that there is a negative pressure present in the lumen of the lung of the fetus as it is delivered at term, and this pressure may serve to ensure that the lung does not dry out completely when absorption of liquid begins.

2.3.3 Colloid oncotic pressure

Colloid oncotic pressure is proportional to the concentration of large impermeant molecules, mainly protein, in a liquid and leads to movement of water and small solute molecules across semi-permeable membranes separating two body compartments, ultimately leading to equalisation of the osmotic pressures in the compartments. Inhibition of active transport in the pulmonary epithelium by cyanide (KCN) has been shown to result in absorption of liquid driven presumably by the colloid oncotic pressure gradient (Olver and Strang, 1974) between alveolar lumen and plasma, a gradient that was estimated to be approximately 20 mmHg. However, absorption was very slow, a fact that was attributed to low alveolar membrane permeability. Whereas the endothelial capillary membrane is considered relatively "leaky" (Normand *et al*, 1971) the alveolar epithelial membrane is "tight" in fetal life, but it appears to undergo a typical perinatal alteration (Taylor and Gaar, 1970; Normand *et al*, 1971; Egan *et al*, 1975) discussed earlier (see 2.3.1). The increase in epithelial pore size around the time of birth (Egan, 1976), as

compared to the pore size of the fetus or older animal, must result in an osmotically driven alveolar liquid absorption during this period. The quantitative importance of this mechanism during normal lung adaptation to breathing at birth remains to be established. The physiological significance of the colloid-osmotic forces in driving at least partially lung liquid absorption was demonstrated in young lambs where hypoproteinemia resulted in a delay in absorption (Cummings *et al*, 1993). Separating active and passive components of liquid absorption has been attempted (Vejlstrup *et al*, 1994) but a complete understanding of the individual contribution of the different driving forces remains to be achieved.

2.3.4 Electrical potential

During fetal life, secretion of Cl^- ions into the lung lumen creates an electrical gradient with a negative pole at the apical side of the pulmonary epithelium (lung liquid side). The mean electrical potential difference measured between lung liquid and plasma was – 3.4 mV (Olver *et al*, 1986). In these experiments, when reabsorption of lung liquid was induced by infusion of adrenaline into the fetal circulation, the potential difference further increased, due to Na^+ passage from lung lumen to plasma. Furthermore, the demonstration that measured ion fluxes exceeded the predicted passive fluxes based on electrochemical gradients only (Koefoed-Johnsen and Ussing, 1953) provided evidence that Cl^- and Na^+ must be actively transported in opposite directions.

2.3.5 Active ion transport

Transport of liquid and solutes across the lung epithelium is not by itself an active process, but is driven by an osmotic gradient resulting from active ion transport mechanisms. As discussed below, the driving force for lung liquid production in fetal life depends on Cl^-

secretion, which appears to be an energy consuming process. Interestingly, liquid absorption of post-natal life is also an ATP consuming process, based on Na^+ transport across the lung epithelium from the luminal part of the epithelium, i.e. from the future air-spaces. Several lines of evidence indicate that both Cl^- and Na^+ transport depend on the same ATP consuming Na^+/K^+ pump in the basolateral membrane of the epithelial cells (Olver *et al*, 1986).

A model summarising active ion transport in the lung epithelium (see Figure 2-1) was first proposed by Olver and co-workers (Olver *et al*, 1986) based on widely accepted similar models of other epithelia (Silva *et al*, 1977; Frizzell *et al*, 1979). An interesting feature of the model is that secretion and absorption are linked (Olver *et al*, 1986; Strang, 1991). The basolateral membrane of the epithelial cell contains the major motor of the ion transport system in the form of an energy consuming Na^+/K^+-ATPase, but importantly also the facilitated $Na^+/K^+/Cl^-$-co-transporter. The Na^+/K^+-ATPase maintains a low level of Na^+ and a negative charge inside the cell and promotes entrance of Na^+ down its electrical and concentration gradient via the $Na^+/K^+/Cl^-$-co-transporter. Cl^- is dragged into the cell up its electrical gradient and once inside the cell it exits passively via specific Cl^- channels in the apical membrane. Na^+ and water follow, giving rise to lung liquid secretion.

A feature of this model is that when Na^+ channels on the apical surface of the epithelial cells (ENaC) are activated, Na^+ enters across the apical surface to be pumped out at the baso-lateral surface by the Na^+/K^+-ATPase. Cl^- and water follow passively and the direction of liquid movement reverses (Brown *et al*, 1983; Olver *et al*, 1986). The key to absorption is the activation of the ENaC, and therefore the fall in resistance to Na^+ entry via this luminal pathway. Resistance to Na^+ passage through the apical membrane may be

regulated either by increasing the number of channels in the membrane or by modulation of channel function and/or structure (Norlin *et al*, 1999; Baines *et al*, 2000). The ENaC, which is considered the central structural element for perinatal lung liquid balance, will be further discussed in Sections 2.5 on lung liquid absorption, in Section 2.6 on developmental modulation of lung liquid and in Section 2.10 on perinatal lung liquid clearance.

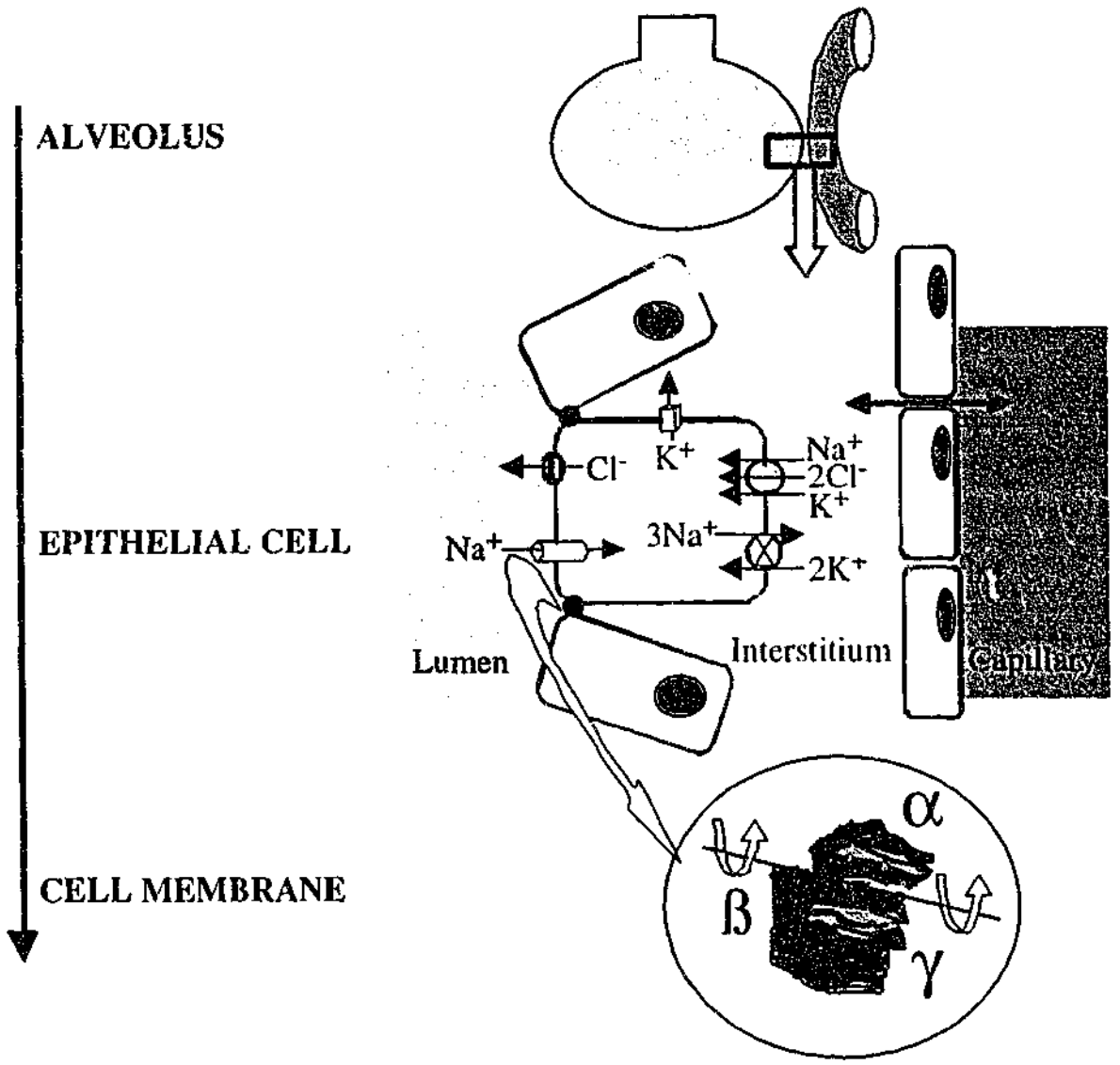

Figure 2-1. *Cellular model of the secretion and absorption occurring in the lung epithelium via Cl^- and Na^+ transport (modified from Olver et al, 1986). The key feature of this two-membrane hypothesis is the different ion transport properties on apical (luminal) and basolateral cell membranes. Na^+/K^+-ATPase is the motor of the ion transport system. Several other ion channels, particularly the $Na^+/K^+/Cl^-$-co-transporter are required for Cl^- secretion. The Cl^- and Na^+ channels in the apical membrane are the key structures for regulation of secretion and absorption (see text).*

2.4 Cl^- and Lung Liquid Secretion

The high Cl^- content of lung liquid (Adams *et al*, 1963a; Adamson *et al*, 1969), and unidirectional ion flux measurements across the pulmonary epithelium (Olver and Strang, 1974), pointed to the likelihood of Cl^- as the ion responsible for liquid secretion. Attempts to quantify secretion rate were first made in goats and guinea-pig fetuses by observation of the liquid meniscus in a glass cannula tied into the trachea (Setnikar *et al*, 1959). Secretion rates (normalised to fetal body weight) of up to 6.7 $ml.h^{-1}.kg^{-1}$ were observed by this method and later values between 12 and 24 $ml.h^{-1}.kg^{-1}$ (Adams *et al*, 1963a) were obtained in similar experiments with an open-ended cannula in the trachea of fetal sheep. However, in these experiments the hydrostatic pressure against which secretion had to occur was not precisely controlled. In an alternative experimental approach, a collecting balloon was placed at the end of the sectioned fetal sheep trachea; intermittent emptying of this balloon provided an estimate of secretion rate of 2 and 10 $ml.h^{-1}.kg^{-1}$ (Enhörning and Adams, 1965).

Introduction of the tracer dilution technique for measurement of lung liquid volume, which averted the need for drainage and siphoning of lung liquid, has been responsible for new insights into the control of lung liquid secretion during fetal life. This technique, which has already been discussed (2.1.1), could be applied under normal physiological conditions in un-anaesthetised fetuses in utero after selectively blocking suspected key components of the secretory mechanism. The procedure allowed an in-depth evaluation of a number of possible cellular mechanisms in lung liquid secretion and absorption and provided further evidence that Cl^- secretion plays the key role in the secretory process. Blocking the Na^+/K^+-ATPase pump in the latero-basal membrane of the lung epithelial cell with ouabain (Boucher and Gatzy, 1983; O'Brodovich *et al*, 1990b) or cyanide (Olver and Strang, 1974)

arrested the process of Cl^- secretion. The role of facilitated Na^+/Cl^- entry into the epithelial cell, and the presence of a specific Cl^- channel at the apical membrane, of which several types of different specificity coexist, further improved our understanding of the cellular mechanisms of lung liquid secretion.

2.5 Na^+ AND LUNG LIQUID ABSORPTION

Olver *et al* first demonstrated the importance of active Na^+ transport for lung liquid absorption across the fetal lung epithelium (Olver *et al*, 1986). Na^+ channels have been demonstrated in most organs and the role of these channels in ion and liquid transport has been elucidated in vitro for the kidneys, the colon, some exocrine glands as well as the lung (Barker and Gatzy, 1993; Krochmal-Mokrzan *et al*, 1993; Duc *et al*, 1994; Voilley *et al*, 1994). Low levels of mRNA of the epithelial sodium channel (ENaC) have been found in the lung of premature rats (Tchepichev *et al*, 1995) and humans (Voilley *et al*, 1994), whereas its expression increases considerably in the perinatal period and subsequently remains high during adulthood (Tchepichev *et al*, 1995). Increased expression of ENaC mRNA in late gestation, and its rapid expression after caesarean birth without labour (Baines *et al*, 2000), suggest that this channel follows a prenatal maturation before its postnatal function is required. The ENaC is known to be the key structure for active Na^+ transport and has been confirmed in multiple experiments to play a key role in clearing alveolar liquid in the adult lung (Effros *et al*, 1986; Basset *et al*, 1987b; Berthiaume *et al*, 1988; Matthay *et al*, 1996). In an attempt to localise the specific liquid absorptive function to specific lung epithelial structures, the ENaC was demonstrated in the membrane of type II pneumocytes (O'Brodovich *et al*, 1991), as their culture in vitro is relatively easy. The testing of type I cells in vitro has not yet been achieved, and the participation of this cell type in lung liquid absorption (or secretion) in vivo consequently is unclear.

Amiloride was described as a potent Na^+ channel blocker through pharmacological studies in different epithelia in animals and humans, particularly of the urinary tract (Benos, 1982). Olver first used amiloride on fetal lung epithelium at the apical or luminal side, i.e. via the airspace of the lung (Olver *et al*, 1986), to demonstrate the importance of the Na^+ channel in adrenaline-induced liquid absorption. Later, more specific Na^+ channel blockers, such as benzamil and phenamil were shown to have a similar but more potent effect (O'Brodovich *et al*, 1991). During fetal life and shortly after birth, if the Na^+ channel is blocked, absorption of liquid from the lumen of the lung does not occur, and secretion of liquid is observed, thereby demonstrating the key role of the Na^+ channel for active liquid movement. However, when the postnatal sheep is several weeks old, blockade of the ENaC no longer causes cessation of liquid absorption, but merely causes a decline in absorption rate (Basset *et al*, 1987a; b; Ramsden and Neil, 1990), suggesting either that a population of amiloride-resistant Na^+ channel develops in the older animal, or that an additional absorptive mechanism that does not involve the Na^+ ion is established with postnatal maturation.

O'Brodovich first demonstrated the crucial role played by the Na^+ channel in adapting the lung for gas exchange immediately after birth (O'Brodovich *et al*, 1990a). In newborn guinea pigs, in which amiloride was administered intra-tracheally to block the Na^+ channel before the newborn took its first breath, post-natal respiratory impairment ensued. This phenomenon was presumed to be caused by flooded lungs, as lung water content was increased in animals treated that way with amiloride. In the cited study, both the control and amiloride-treated animals survived the first 4 postnatal hours until termination of the experiment. However, both groups of animals were maintained on artificial ventilation and it is therefore not known whether the amiloride-treated animals would have survived

without ventilatory assistance. Using an elegant Na^+ channel knock-out model, subsequent work has confirmed the importance of the ENaC for postnatal adaptation of the lung (Hummler *et al*, 1996). The knock-out animals had a normal fetal outcome and normal lung structure, supporting the view that ENaC function is not essential for fetal development. However, knock-out animals did not have ventilatory support and they died after birth with a high water content in the lung. This finding suggested that death resulted from a failure of absorption of liquid from the lung, or from failure to terminate lung liquid secretion. It is also of importance to note that death did not occur immediately after birth in the ENaC-deficient mice; instead they survived for up to 40 hours. A possible explanation for survival of these animals for many hours is that secretion of liquid could have persisted in the knock-out animals, adding to the liquid already present in the lung lumen at the time of delivery and eventually exceeding the level at which respiratory failure occurs.

It is now clear that the ENaC is made up of three distinct homologous subunits, alpha, beta and gamma (Canessa *et al*, 1994). All three channel subunits have been cloned recently, providing a molecular definition of this amiloride-sensitive Na^+ channel (Garty and Palmer, 1997). In vitro, expression of the subunits beta and gamma alone in the Xenopus laevis oocyte did not generate a channel activity (Canessa *et al*, 1994; McDonald *et al*, 1995), as demonstrated by absence of electrogenic Na^+ transport, whereas the alpha subunit alone was able to generate a weak channel function. Co-expression of the gamma and alpha units generated more channel activity than normal. Interestingly rat subunits were replaced by human subunits without functional loss of the channel (McDonald *et al*, 1995) demonstrating the occurrence of very similar Na^+ channels between species. However, when beta and gamma subunits are present but the alpha subunit is absent, channel function is completely disrupted (Canessa *et al*, 1994; McDonald *et al*, 1995). As

to its function in vivo, both subunits, alpha as discussed earlier (Hummler *et al*, 1996; Hummler *et al*, 1997) and beta (McDonald *et al*, 1999) have been knocked out genetically in mice, without apparent damage to fetal development. However, neonatal survival curve was very similar in both knock-out models and death occurred, not immediately, but up to 40-50 hours after normal delivery. In the alpha subunit deficient mice death was clearly attributable to lung liquid drowning, whereas in the beta subunit deficient mice the suggested cause of death was electrolyte imbalance and in particular hyperkalemia. The pups in the beta subunit deficient mice were clinically indistinguishable from their littermates until death occurred and, contrary to the alpha subunit deficient mice, they did not show any signs of respiratory distress. However, their postnatal lung wet to dry weight ratio of ~ 6.5 (McDonald *et al*, 1999) was significantly higher than in the lungs of littermates, and interestingly not far from the ratio reported for the alpha subunit deficient lungs (wet/dry ratio of 7.1 – 7.9) (Hummler *et al*, 1996; Hummler *et al*, 1997).

2.6 Developmental Modulation of Secretion and Absorption

Gestational age and lung maturation are the main determinants of neonatal morbidity and mortality. Research directed at understanding the reason for this association has concentrated on pulmonary surfactant, ultimately leading to the establishment of replacement therapies. However, the widespread clinical use of surfactant replacement has not led to abolition of respiratory disease, pointing to the likelihood that failure of other maturational processes is a factor in the development of neonatal lung disease. Most commonly cited factors playing a role in lung maturation are included in Table 2-5.

A key motivation for the studies performed in this Thesis is that failure to clear lung liquid may contribute to the neonatal respiratory disease. As lung liquid absorption is achieved

via epithelial Na^+ transport, it is important to consider the maturation of the central structural component of this transport mechanism, the ENaC, which is synchronised with the approach of term, just as occurs in the surfactant system. Modulation of this channel, and therefore of Na^+ transport and liquid reabsorption from the lung, may occur at two different levels, first through control of expression and synthesis of ENaC and also by functional regulation of the channel.

Table 2-5. *Factors influencing lung maturation*

Stimulation	**Inhibition**
Steroids	Maternal diabetes
Thyroid hormones	Male sex
TRH	Transforming growth factor-beta
NO	
cAMP	
Gamma-interferon	
Prolactin	

Table modified from Gross, 1990

2.6.1 Expression and synthesis of the ENaC

Steroids

Fetal glucocorticoid concentrations rise exponentially at the end of gestation and during labour (Bassett and Thorburn, 1969; Kitterman *et al*, 1981a; Wallace *et al*, 1995; Lines *et al*, 1997). The functional significance of this rise was first demonstrated by Liggins (Liggins, 1969) who reported enhanced survival of premature sheep treated with steroids antenatally. Since that time numerous studies have demonstrated that steroids accelerate morphological and physiological maturation of the fetal lung (Kikkawa *et al*, 1971; Mescher *et al*, 1975; Venkatesh and Katzberg, 1997; Stokes and Sigmund, 1998). As a

result, corticosteroids have become the routine treatment for mothers at high risk for preterm delivery, leading to a decline in neonatal respiratory morbidity (Crowley, 2000).

The effect of steroids on neonatal lung function has been classically attributed to maturation of the surfactant system (Gross, 1990) but some research has investigated the effect of steroid on the maturation of the ENaC at a molecular level (Venkatesh and Katzberg, 1997; Geller *et al*, 1998; Stokes and Sigmund, 1998) and to a lesser extent at a functional level (Cassin *et al*, 1994; Wallace *et al*, 1996b). Experiments in which cortisol (Kitterman *et al*, 1981a; Liggins *et al*, 1988; Schellenberg *et al*, 1988; Kindler *et al*, 1993; Wallace *et al*, 1995; Norlin *et al*, 1999) or aldosterone (Kindler *et al*, 1993) was given to the fetus resulted in reduced liquid secretion, or induced absorption, strongly suggesting a potent steroid-mediated maturation of the ENaC.

In the guinea-pig lung the expression of ENaC is developmentally regulated, with a peak in late gestation and early neonatal life (Baines *et al*, 2000). Compared to animals that delivered vaginally at term, expression of the alpha chain of the ENaC after caesarean section is delayed until after delivery and could be blocked by cortisol synthesis inhibition. These findings suggest that one of the beneficial effects of steroids may also be the result of their influence on lung liquid absorption. Clinical practice is in line with this view, as surfactant replacement therapy in neonatal respiratory distress does not result in immediate and complete recovery, suggesting that a slow process, such as water clearance, may be involved. The recent technical possibilities of magnetic resonance imaging in newborns with RDS has lent support to this possibility in that it shows a significantly increased lung water content in RDS lungs compared to similarly-aged control premature infants without RDS (Adams *et al*, 2000a). Thus, immaturity of liquid clearance emerges as an important

but little recognised cofactor of respiratory morbidity in neonates, particularly in the premature infant (O'Brodovich, 1996).

Thyroid hormones

Evidence has accumulated that fetal lung maturation is influenced by thyroid hormones. Drug-induced (Kerepesi and Rady, 1984), surgical (Barker *et al*, 1988) or genetic (deMello *et al*, 1994) fetal hypothyroidism delays lung maturation in rats, sheep and mice. The positive effect of thyroid hormones on lung maturation was suggested by a combined prenatal treatment with thyrotropin-releasing hormone (TRH) and steroids in lambs (Schellenberg *et al*, 1988). Based on these findings the same group presented in 1988 the first data on human prenatal treatment with TRH and steroids in abstract form (Liggins *et al*, 1988). However, contrary to expectation, when this prenatal treatment was tested in several clinical randomised trials, it did not reduce the risk of neonatal respiratory disease but demonstrated considerable adverse effects (Crowther *et al*, 2000).

Several reports continue to attribute an important role to thyroid hormones in lung maturation and possibly to expression of the ENaC. Thus fetal thyroidectomy profoundly suppresses the adrenaline-inducible Na^+ absorption, an effect which returned to normal under fetal thyroid hormone replacement (Barker *et al*, 1988; Barker *et al*, 1990a; Barker *et al*, 1990b). In addition, enhanced pulmonary beta-adrenergic receptor synthesis occurs after thyroid hormone treatment in rats (Whitsett *et al*, 1982). These and similar findings have led to the conclusion that thyroid hormones exert a maturational effect, probably via a synergistic action with steroids, and possibly arginine vasopressin (AVP), on the synthesis or maturation of the ENaC (Barker *et al*, 1990a; Barker *et al*, 1991; Cassin *et al*, 1994).

2.6.2 Functional regulation of the ENaC

It is not clear how the Na^+ transport mechanism is turned on in the lung in the perinatal period, nor is it known what then maintains Na^+ transport activity throughout postnatal life. Is it a new trigger (substance) released by the birth process activating an ENaC matured by steroids and thyroid hormones, or could it be that the loss of a fetal inhibitory mechanism on the Na^+ transport mechanism results in release of a previously blocked ENaC? An inhibitor of the sodium pump, possibly originating from the placenta, has been suggested (Hilton *et al*, 1996), although its composition and its biosynthetic pathway remain unknown. Several other endogenous and exogenous substances can affect Na^+ transport function, mostly by inhibition or augmentation of ENaC function.

Inhibition of the ENaC: enhancement of secretion

Blockade of the ENaC with amiloride is capable of restoring secretory function in the lung of the fetus and early newborn, although not in the older newborn or adult (Ramsden and Neil, 1990). Intra-luminal administration of prolactin has been reported to cause a 3.6 fold increase in lung liquid secretion (Perks and Cassin, 1982). In the same report (Perks and Cassin, 1982), administration of intravenous saline to anaesthetised and exteriorised fetal goats increased secretion rate significantly. Finally, the triphosphate nucleotides, adenosine triphosphate (ATP) and uridine triphosphate (UTP), have been reported to induce Cl^- secretion across the fetal lung epithelium in vitro (Barker and Gatzy, 1998). This effect was also demonstrated in the upper airway epithelium of adult human in vivo by the trans-epithelial potential difference resulting after superfusion of these nucleotides (Knowles *et al*, 1991). Stimulation of Cl^- secretion by nucleotides was found to be equipotent for ATP and UTP and dose-related with maximal effective concentration around 10^{-4} M (Knowles *et al*, 1995a). When Na^+ absorption was blocked with amiloride, UTP superfusion of the

nasal epithelium in healthy adult humans activated Cl^- secretion; this effect was not observed in the absence of amiloride blocking the Na^+ channel. These findings confirm that Cl^- secretion and Na^+ absorption are also closely linked in adult upper respiratory tract epithelium, and that Na^+ flow into the cell via ENaC is favoured over the $Na^+/K^+/Cl^-$ co-transporter (see Figure 2-1).

Activation of the ENaC: induction of absorption

Beta-adrenergic stimulation. The number of beta-receptors in the lung epithelium increases with gestation (Giannopoulos, 1980; Whitsett *et al*, 1981). The increased steroid and thyroid hormone levels near term may cause this increase in the number of beta-receptors (Cheng *et al*, 1980; Whitsett *et al*, 1980; Ballard, 1983) and could also be associated with their physiological activation (Barker *et al*, 1990b). This maturational effect may explain the observation that intravenous infusion of epinephrine or isoproterenol at doses varying from 0.1-1.0 $\mu g.kg.min^{-1}$ caused a decrease in secretion or initiation of absorption in preterm fetal sheep that was gestational-age dependent (Walters and Olver, 1978; Brown *et al*, 1983), an effect which was reversed by the beta-blocker propranolol. Further research provides clear evidence that in the mature fetal lung, active amiloride-blockable Na^+ absorption can be induced by adrenaline (Olver *et al*, 1986; Perks and Cassin, 1989; Ramsden *et al*, 1992). There is also evidence that beta-adrenergic stimulation acts via release of cAMP as a secondary intracellular messenger (Walters *et al*, 1990). The physiological importance of this trigger is suggested by the inverse correlation between lung liquid secretion and endogenous circulating adrenaline levels which are low during early gestation (Comline and Silver, 1961; Buhler *et al*, 1978) before rising to high values during the last stage of labour (Brown *et al*, 1983) when reduction of lung liquid volume improves postnatal gas exchange (Berger *et al*, 1996).

Despite considerable evidence in favour of adrenaline playing an important role in the perinatal initiation of lung liquid reabsorption, beta-adrenergic blockade in fetal rabbits has been reported not to prevent liquid clearance at birth (McDonald *et al*, 1986). A similar finding was reported in fetal sheep where evidence was presented that liquid secretion fell during spontaneous labour despite beta-blockade (Chapman *et al*, 1994). These two studies raise the interesting possibility that there may be mechanisms in addition to beta-adrenergic stimulation that promote lung liquid clearance in the perinatal period. However, there are reasons to question whether the findings of these two studies prove that adrenaline plays no role in the process of lung liquid clearance. First, in one of the studies, many fetuses entered labour at an age less than 140 days (range from G130 to G145) (Chapman *et al*, 1994), well before normal term (G147) in the sheep. Since it has been shown that adrenaline mediates its action through beta-receptors, and the number of these receptors increases markedly close to term (Whitsett *et al*, 1981), a lack of effect of beta-blockade in a relatively immature group as a whole might not be surprising. The likelihood that the animals studied were immature, at least in terms of the adrenaline-stimulated reabsorptive mechanism, is supported by the fact that adrenaline merely blocked secretion and did not initiate reabsorption as it does in the mature fetus (Walters and Olver, 1978; Brown *et al*, 1983). Second, although there was a fall in secretion rate during labour in the animals studied, no statistically significant reabsorption was observed (Chapman *et al*, 1994); that is, adrenaline-mediated reabsorption had not been initiated in animals studied, and the lack of an effect of β-blockade would be expected.

Arginine vasopressin (AVP). Several studies report that AVP decreases lung liquid secretion in fetal sheep (Perks and Cassin, 1982; Ross *et al*, 1984; Perks and Cassin, 1985b; a; 1989; Wallace *et al*, 1990). Similar to adrenaline, AVP levels are elevated at

birth (Stark *et al*, 1979) and this agent might therefore contribute to liquid clearance in the perinatal period. Again similar to adrenaline, a maturational process occurs as the effect of AVP on secretion increases progressively towards the end of gestation (Perks and Cassin, 1985a). AVP has been shown to induce liquid absorption in the lung in vitro (Goodman *et al*. 1984), and when infused in vivo during late gestation it reduces secretion rate, and this effect occurred in the absence of a change in adrenaline levels (Wallace *et al*, 1990). Thus AVP directly affects secretion and the effect is amiloride-blockable in lambs (Cassin and Perks, 1993; Hooper *et al*, 1993b) and guinea-pigs (Perks *et al*, 1993), strongly suggesting that AVP exerts its effect via activation of the ENaC. In the fetal sheep well before term (G125), again similar to adrenaline, AVP by itself appears unable to alter lung liquid secretion (Cassin *et al*, 1994). However, at G125 when combined with steroids and thyroid hormones, AVP was able to reduce secretion or to induce absorption (Cassin *et al*, 1994), indicating a physiologically significant interaction, probably via activation of the ENaC after its boosted expression by steroids and thyroid hormones (see 2.6.1).

In human kidney medullary cells, stimulation of AVP V2 receptors increased cAMP levels (Guillon *et al*, 1982). These similarities between AVP and adrenaline suggest the two hormones act via the same pathway, possibly via the release of intracellular cAMP. AVP may therefore present one of the alternate pathways of stimulation of the ENaC discussed earlier.

Adenosine 3',5'-cyclic monophosphate (cAMP). Substances increasing intracellular cAMP in type II pneumocytes in vitro stimulate active liquid transport (Goodman *et al*, 1984; Cott *et al*, 1986), and a similar effect is found in vivo in rats and sheep (Olver *et al*, 1987; Walters and Ramsden, 1987; Walters *et al*, 1990; Berthiaume, 1991; Berthiaume *et al*,

1999). Similar to adrenaline, the effect of cAMP on lung liquid secretion increases with gestational age (Walters *et al*, 1990). The effect can be blocked by amiloride, suggesting that cAMP ultimately acts via the ENaC. Evidence suggests that cAMP modulates the ENaC by the same pathway affected by adrenaline, but it bypasses the beta-receptor and adenylate cyclase (Goodman *et al*, 1984; Walters and Ramsden, 1987; Walters *et al*, 1990).

At least two classes of agonist, beta-stimulants (Walters and Olver, 1978; Walters and Ramsden, 1987) and AVP (Guillon *et al*, 1982; Coleman and Kennedy, 1985) are known to activate or release cAMP and to induce active Na^+ absorption. A common activation pathway of the ENaC via cAMP as a second messenger may be inferred (Walters *et al*, 1990). In addition, aminophylline and other phosphodiesterase inhibitors, which are known to stimulate cyclo-oxygenase and consequently cAMP production, have been shown to increase liquid absorption in vitro (Goodman *et al*, 1984) and in vivo in rats (Berthiaume *et al*, 1999). Finally, salmeterol, which increases intracellular cAMP when instilled into rat and sheep lung, also stimulated lung liquid clearance (Berthiaume *et al*, 1999).

Some uncertainty persists on the molecular pathway between cAMP and ENaC activation, although in the alveolar type II cell in vitro, protein kinase A activates the Na^+ channel (Yue *et al*, 1994). However, in this in vitro model, the Na^+ channel has a low amiloride sensitivity, corresponding more to an adult type channel, than the perinatally active ENaC. It is interesting to note that an endogenous inhibitor of cAMP, found in most organs including the lung, is stimulated by insulin (Wasner *et al*, 1993) and may explain in part the increased incidence of respiratory disease in term babies of diabetic mothers (Robert *et al*, 1976) generally attributed to delayed surfactant maturation. However the higher incidence of respiratory disease occurs in the presence of reliable indicators of lung

maturity (Dunn *et al*, 1981; Ma *et al*, 1997; Piazze *et al*, 1999) and may well reflect delayed liquid absorption.

Loop diuretics. Loop diuretics, such as furosemide and bumetanide, seem to affect lung liquid transfer by a mechanism other than diuretic (Demling and Will, 1978). It was hypothesised that their effect in reducing secretion may result from inhibition of the facilitated $Na^+/K^+/Cl^-$ co-transport (Cassin *et al*, 1986; O'Brodovich *et al*, 1990b) at the basolateral membrane of the pulmonary epithelial cell. Indeed, Cl^- and liquid secretion are inhibited by loop diuretics applied to the basolateral surface of the lung epithelium in vitro (Shabarek *et al*, 1994; Clerici *et al*, 1995) and in vivo in sheep (Cassin *et al*, 1986) and guinea-pigs (Thom and Perks, 1990). A randomised controlled human trial tested orally administered furosemide in an acute setting of newborns with suspected "wet lung" at birth, but found no significant effect on duration of tachypnea (Wiswell *et al*, 1985). However, in bronchopulmonary dysplasia, a condition of the premature infant where increased lung liquid or oedema is hypothesized, a systematic review concluded that furosemide was able to improve lung function acutely when given either systemically or by pulmonary nebulization (Brion *et al*, 2000). Diuretics are indeed now classically used in neonatal chronic lung disease, probably in up to 50% of cases, but still little is known about long term effects of this treatment on the lungs.

Other agents. Several other agents have been observed to reduce secretion or induce absorption. Basically any substance affecting ATP consuming processes may affect lung liquid absorption (Hilton *et al*, 1996). Attention has focussed on agents that impact on pulmonary vascular resistance; oxygen (Dawes *et al*, 1952), prostaglandins (Cassin, 1987) and nitric oxide (Abman *et al*, 1990; Cornfield *et al*, 1992).

The rise in oxygen levels at birth appears not to stimulate Na^+ absorption in physiological studies and in fact, oxygenation of chronically instrumented fetal sheep, via liquid ventilation with a solution containing haemoglobin, was reported to increase lung liquid secretion (Round *et al*, 1999). A complementary observation was made in vivo during fetal hypoxia, when liquid secretion diminished compared to normoxia (Hooper *et al*, 1988; Hooper *et al*, 1993b). Acidemia accentuated the effect of hypoxia (Wallace *et al*, 1996a). These effects of hypoxia did not appear to be secondary to the stress hormone adrenaline, as beta-blockade did not modify secretion rate. By contrast, in vitro studies of cultured lung epithelium exposed to different oxygen levels suggested activation of Na^+ absorption by oxygen (Barker and Gatzy, 1993). However, this effect of elevated oxygen in vitro is not directly in contradiction with physiological studies, as it occurred over a longer time period than the in vivo studies. Increased mRNA levels demonstrated that this long-term effect resulted from increased Na^+ channel synthesis (Pitkanen *et al*, 1996; Baines *et al*, 2000).

Intra-luminal nitric oxide (NO), one of the major mediators of the transitional pulmonary circulation, has also been demonstrated to reduce secretion rate in fetal sheep (Cummings, 1997). This effect was accompanied as expected by an increased pulmonary blood flow due to vaso-dilatation of the pulmonary vasculature. The effect on secretion rate was Na^+-channel independent, as blocking the channel with amiloride or benzamil did not reverse it. As reduced secretion, but never absorption, was induced by NO, it is possible that NO acts on Cl^- secretion only. A similar effect resulted from infusion of cyclic guanylate monophosphate (cGMP) to the pulmonary circulation of the fetal sheep (Kabbani and Cassin, 1998), suggesting the hypothesis that NO might act through the guanylate cyclase intracellular pathway.

Other agents, such as acetylcholine, prostaglandin D2 or leukotriene-blockers, have been shown to decrease lung liquid production in fetal sheep (Cummings, 1995) and their substantial rise in concentration during labour and birth make them ideal candidates for reducing liquid secretion before birth (Kitterman, 1984). Because these agents reduce pulmonary vascular resistance by different mechanisms, it has been suggested that their mode of action on liquid secretion is via increased pulmonary blood flow (Cummings, 1995). The other potential pathway, via cAMP, has been investigated by direct intra-luminal application of prostaglandin E2 (Wlodek *et al*, 1998; Berthiaume *et al*, 1999) and by testing the known effect of beta-stimulation on liquid absorption after inhibition of prostaglandin synthesis with indomethacin in the adult rat and sheep (Berthiaume *et al*, 1999). These studies, together with experiments involving indomethacin blockade of prostaglandin synthesis in the fetal sheep (Kitterman *et al*, 1981b), suggest that prostaglandins do not play a major role in peri- and postnatal Na^+ absorption.

Interestingly, an effect of prostaglandins on Cl^- secretion appears likely from fetal sheep studies in vivo (Cassin, 1984; Stevenson and Lumbers, 1992; Wlodek *et al*, 1998) where indomethacin decreased lung liquid secretion rate significantly, just as it does in vitro on human pulmonary epithelium. Consistent with prostaglandins affecting lung liquid secretion, prostaglandin inhibitors are known to reduce amniotic fluid volume (Wlodek *et al*, 1994) which in part results from lung liquid.

Physical influences. After birth the human newborn temperature may fall as much as 2 - 3 °C (Adamsons and Towell, 1965). It has been hypothesized that such a fall in temperature may trigger liquid absorption at birth (Garrad-Nelson and Perks, 1990). However, since a pronounced fall in temperature would be expected to reduce the activity of metabolic

processes consuming ATP, a reduction in lung liquid absorption would also be expected, as it too is energy driven, being dependent upon the same Na^+/K^+-ATPase that drives Cl^- secretion (see section 2.3.5). An argument against a major role of temperature in modulating lung liquid secretion or absorption is that in today's delivery room practice, central temperature of the human newborn is monitored and supported carefully, resulting in falls of only 0.5 – 1.0 °C (Johanson and Spencer, 1992; Christensson *et al*, 1993) and only exceptionally leading to hypothermia with falls of more than 1.5 - 2 °C. In addition, in clinical practice, hypothermic babies at birth are not known to present a higher incidence of "wet lung", although no systematic review can be found on the subject.

Lung distension may play an important role in the regulation of secretion. Lungs of human newborns with an obstructed trachea are abnormally-distended and liquid-filled (Potter and Bohlender, 1941; Carmel *et al*, 1965). Under these conditions, in the lamb fetus, lung liquid volume ultimately reaches a plateau (Hooper *et al*, 1993a; Keramidaris *et al*, 1996), indicating either that distension itself, or the resulting increase in intra-luminal pressure, leads to a reduction of secretion to zero, or to the initiation of absorption at a rate that balances secretion rate. Expansion itself appears as an appealing trigger for liquid absorption, since expansion of the lung with the first breath at birth is an obligatory step in newborn adaptation to air breathing, and could then be seen as the key trigger in removal of liquid from the lung. There is evidence that static lung distension does indeed reduce secretion, and in some cases results in absorption (Egan, 1976; Perks and Cassin, 1985b; Ramsden and Neil, 1993; Vejlstrup *et al*, 1994; Garrad-Nelson and Perks, 1996; Kojwang and Perks, 1996). The question, however, as to whether distension induces active absorption or acts via hydrostatic pressure (see Section 2.3.2) and possibly epithelial permeability (see Section 2.3.1) is not answered clearly yet, as some experimental models

may have been compromised by hypoxia which itself reduces secretion as outlined earlier. Furthermore, some evidence is not consistent with expansion leading to reduced secretion or to absorption. Dickson and Harding (1987) were unable to detect changes in secretion rate after either a sudden reduction or increase of lung volume. In these experiments the fetuses studied were more than 1 week before term and it is therefore possible that the Na^+ absorptive mechanism was still immature. In addition, in the experimental protocol, volume was altered once only before returning rapidly to normal; it is possible that a more prolonged or repetitive exposure to increased or decreased lung volume, more equivalent to normal breathing, might have led to altered secretion or to absorption of liquid from the lung.

A recent study on murine lung epithelial cells that were rhythmically stretched for more than 30 to 60 minutes reported an increased Na^+/K^+-ATPase activity (Waters *et al*, 1999). As in vivo active Na^+ absorption depends on Na^+/K^+-ATPase activity, this finding again focuses attention on lung stretch as a potential trigger for initiating absorption after birth. Such a possibility is supported by the strong evidence that stretch induced by a small hydrostatic pressure gradient increased Na^+ transport across the epithelium of the rabbit bladder (Lewis and de Moura, 1982) and frog skin (Nutbourne, 1968).

2.7 Control of Resting Lung Volume

In the fetal sheep, the volume of lung liquid and its rate of secretion have been studied from 70 days gestation until term at 147 days (Olver *et al*, 1981). This work showed that at an average age of 74 days gestation, fetuses weighed 231g and had a luminal volume approximating 1 ml. During the last third of fetal development in the lamb, when most volume measurements have been made, it was generally accepted until recent years that

approximately 30 ml.kg^{-1} body weight of liquid fills the lumen of the lung, approximating functional residual capacity in the newborn (Normand *et al*, 1971; Scarpelli *et al*, 1975; Olver *et al*, 1981; Bland *et al*, 1982; Dickson and Harding, 1989). By contrast, there have recently been several reports (Hooper and Harding, 1995; Harding and Hooper, 1996; Lines *et al*, 1997) that the volume of liquid in the lung in late gestation vastly exceeds postnatal functional residual capacity, with values as high as 50 ml.kg^{-1} being attained on the day preceding labour.

The issue of production has been discussed earlier (see Section 2.2), but briefly it has been known for almost 20 years that virtually no liquid enters the lungs via the trachea, which acts as a "non-return valve", essentially imposing unidirectional flow through the larynx, unless hypoxia induces "gasps" (Brown *et al*, 1983). Thus, in the healthy fetus, liquid enters the lung only by secretion across the alveolar wall.

Only two potential pathways exist through which liquid may leave the lungs; through the epithelium itself, or via the trachea. Mechanisms giving rise to active and passive trans-epithelial ion and liquid movements have been discussed already, including their role in lung liquid volume regulation during fetal life and their contribution to lung liquid clearance : postnatal life (see Section 2.3). It might be reiterated in this context of trans-epithelial liquid transfer that it is now well established that active Cl^- secretion plays the essential role during fetal life whereas active Na^+ absorption activity starts only some 1 - 2 hours before birth, emphasising its essentially postnatal function.

During late fetal life, lung liquid flows out of the upper airway continually (Lanman *et al*, 1971; Alcorn *et al*, 1977; Perks and Cassin, 1982; Dickson *et al*, 1987; Dickson and

Harding, 1989; Moessinger *et al*, 1990; Harding and Liggins, 1991; Barker and Gatzy, 1993; Nardo *et al*, 1995); measurements suggest that the larynx represents about 80% of the total resistance to outflow (Harding *et al*, 1986). Outflow of liquid through the trachea has been shown to be considerably less during periods of apnea than during fetal breathing (Harding *et al*, 1984b; Dickson *et al*, 1987), demonstrating that laryngeal resistance is lower when the fetus is breathing. In fetal sheep, phasic laryngeal abduction was evidenced in the low voltage electrocortical state, while mild tonic adduction was present in the high voltage state although the glottis remained open (Harding *et al*, 1980). Secretion rate did not change during different electrocortical states but tracheal flow was shown to increase at the start of the low voltage state in which the fetus breathes (Dickson *et al*, 1987).

While the evidence shows that the larynx is differentially activated across electrocortical states, there is no direct evidence to support an active role of the larynx in the control of lung liquid volume. An acute increase of 25%, or a reduction of 50%, in lung liquid volume does not result in alterations to laryngeal activity that would act to restore volume to its control level; instead volume is restored in a passive manner after these disturbances (Dickson and Harding, 1987). Thus, on the basis of this evidence, the upper respiratory tract has mainly a passive role in directing liquid flow, preventing influx and maintaining lung liquid composition. Active control of resistance by the larynx, and therefore of fetal lung volume, would imply that a positive pressure gradient is maintained between lung liquid and amniotic fluid. Measurements of small pressure differences prove very difficult in an environment with large background changes in the amniotic fluid pressure. However, several authors measured a small positive pressure in the lung compared with amniotic fluid of around 2 to 4 cm H_2O (Vilos and Liggins, 1982; Fewell and Johnson, 1983) and in one situation even up to 7 cm H_2O (Müller-Tyl *et al*, 1981). Whether this pressure requires

defence, however, is open to question, since it has been reported that curarisation of the fetus does not diminish the positive tracheal pressure, suggesting that this pressure may result from secretion acting against the resistance of the upper airways (Vilos and Liggins, 1982). One piece of evidence that suggests active laryngeal control of volume is the relative lung hypoplasia resulting from fetal tracheostomy (Fewell *et al*, 1983), but this may also simply result from reduced lung liquid volume consequent upon loss of the resistance resident in the larynx and upper airway.

A final piece of evidence suggesting that lung liquid volume is not actively defended comes from the observation that reduction of the extra-thoracic amniotic fluid compartment by drainage leads to reduced lung volumes (Dickson and Harding, 1989). Thus, volume appears to be controlled by a fine balance of pressures between intra- and extra-thoracic liquid, rather than by control of laryngeal resistance. This hypothesis explains the observation that hypoplastic lungs result from renal absence (Potter and Bohlender, 1941), since the kidneys are known to contribute to amniotic fluid production (Brace, 1986).

2.8 Lung Liquid and Lung Growth

A large body of evidence suggests that lung liquid volume has a major influence on lung growth. The evidence for volume, and hence lung expansion, being the essential component for lung growth is derived from clinical and experimental conditions where lung growth and development are altered when volume is either abnormally increased (Alcorn *et al*, 1977; Moessinger *et al*, 1990; Hooper *et al*, 1993a; Nardo *et al*, 1995) or reduced (Alcorn *et al*, 1977; Dickson and Harding, 1987). It is interesting to note here that a functional Na^+ channel, although absolutely essential for the post-natal lung, is not

necessary for normal fetal lung growth indicating that, in contrast to the Cl^- channel, the Na^+ channel must not play an essential role in fetal lung volume regulation (Hummler *et al*, 1996; McDonald *et al*, 1999).

Considering the rate of growth of the lung, more than 99% of the secreted lung liquid must flow from the lungs through the trachea. Part of the liquid flows directly into the amniotic fluid, and the remainder is swallowed (Harding *et al*, 1984a) before it ultimately reaches the amniotic or allantoic sacs as urine. Thus less than 1% of the liquid produced by the lung remains inside the lung to occupy the space created by growth, whereas the rest contributes to amniotic fluid, almost in equal parts with fetal urine (Kullama *et al*, 1994). Through this contribution to amniotic fluid production, lung liquid appears also to play an important role for lung growth in that it guarantees the external space necessary for expansion by avoiding compression of the fetus and its thoracic wall.

2.9 Lung Liquid Balance: Patho-Physiological Aspects

Pathologies affecting lung liquid secretion and absorption do not lead to fetal death, nor do they seem to impede overall fetal growth and development, with the exception of lung growth as already discussed (see 2.8). However, these pathologies may have profound impact upon the newborn and adult, sometimes leading to a serious clinical outcome. Pathologies may present very differently depending on the type and degree of the defect.

2.9.1 Reduced fetal lung volume

Fetuses lacking thoracic space for an increase in lung volume, as occurs in congenital diaphragmatic hernia (Moessinger, 1990), or in experimentally-induced situations (De Lorimier *et al*, 1967; Harrison *et al*, 1980; Pringle *et al*, 1984) where abdominal content is

made to take up some of the thoracic space, are born with lung hypoplasia (abnormally small and immature lungs). Experimental reduction of the extra-thoracic amniotic space by continuous drainage of amniotic fluid has also been shown to result in reduced lung volumes (Moessinger *et al*, 1983; Moessinger, 1986; Moessinger *et al*, 1986; Dickson and Harding, 1989), a picture found in Potter syndrome (Potter and Bohlender, 1941), where absent kidneys cause oligo-hydramnios and lung hypoplasia. Similarly, a chronic loss of lung liquid can induce lung hypoplasia as evidenced experimentally by continuously draining lung liquid (Alcorn *et al*, 1977).

Potter's oligohydramnios sequence, or Potter syndrome (Potter and Bohlender, 1941), is one of the more frequent multiple congenital anomaly syndromes with an incidence of 1:3000 in human newborns and a poor vital prognosis. Anuria, mainly secondary to renal agenesis, but sometimes of other aetiology, is at the origin of a severe oligohydramnios, nowadays usually diagnosed by ultrasound in human pregnancies, and leading to a typical facies, postural contractions and severe lung hypoplasia. Shortly after birth, newborns die from respiratory and renal insufficiency.

2.9.2 Increased fetal lung volume

Early observations that congenital upper airway atresia led to over-distension and increased size of the lungs below the level of the blockage were reported in a number of studies (Potter and Bohlender, 1941; Jost and Policard, 1948; Carmel *et al*, 1965; Lanman *et al*, 1971) and also demonstrated experimentally as discussed in Section 2.2. The effect of distension on growth of the pulmonary parenchyma, however, was not recognized until Alcorn (Alcorn *et al*, 1977) demonstrated clear histologic evidence of altered tissue structure after chronic distension. More recently, a number of studies have focussed on

interventions to distend fetal lungs to recover lung growth in pathologies with expected or diagnosed lung hypoplasia such as congenital diaphragmatic hernia (Moessinger *et al*, 1990; Hooper *et al*, 1993a; Nardo *et al*, 1995; Harrison *et al*, 1996).

2.9.3 Chloride channel defect

Cystic fibrosis

Cystic fibrosis (CF) is a recessive genetic disease resulting from abnormal lung epithelial Cl^- transport (Dinwiddie, 2000). The Cl^- channel involved in CF, a cAMP-dependent Cl^- channel, is distinct from the Cl^- channel responsible for lung liquid secretion in fetal life. A defect of the specific CF Cl^- channel leads to insufficient liquid secretion in postnatal life and this is coupled with excessive Na^+ absorption (Boucher *et al*, 1988; Dinwiddie, 2000). Together these defects in ion transport are believed in the airway to be the cause of thick secretions resulting finally in congestion, pulmonary infection and chronic airway disease. Whereas until recently pharmacotherapy of the CF lung was mainly symptomatic, and largely based on antibacterial treatment, new understanding of ion transport mechanisms now offers scope for better targeting of ion transport mechanisms (Knowles *et al*, 1995a; Knowles *et al*, 1995b) with the goal of preventing lung damage. Some of the possible drugs now under clinical testing include amiloride which may reduce excessive Na^+ and liquid absorption, and uridine triphosphate which stimulates Cl^- secretion (see Section 2.6.2).

2.9.4 Sodium channel defect

Pseudohypoaldosteronism type 1

This autosomal recessive disease is characterised by a lack of Na^+ channel activity, due to a loss-of-function mutation on the genes encoding for one of the ENaC subunits (Chang *et*

al, 1996; Strautnieks *et al*, 1996b). A mutation on the gamma subunit was identified in pseudohypoaldosteronism type 1 families (Strautnieks *et al*, 1996a) and more recently a disruption of the beta subunit in mice created a similar phenotype (McDonald *et al*, 1999). The effect of this ENaC dysfunction is a salt wasting syndrome characterised by hyponatremia and hyperkalemia resulting in maximal feedback stimulus on aldosterone secretion due to the absence of target effect. The disease has the signs and symptoms of hypoaldosteronism, but with very high aldosterone levels, and is therefore called Pseudo-hypoaldosteronism. One of the clinical features investigated recently is increased airway surface liquid accompanied by recurrent episodes of chest congestion especially in younger patients (Kerem *et al*, 1999), conditions similar to those displayed by beta-subunit deficient mice (McDonald *et al*, 1999).

Pseudohyperaldosteronism (Liddle's Syndrome)

In contrast to the disease described above, the autosomal dominant condition named Liddle's syndrome results from a gain-of-function mutation on the beta subunit of the ENaC (Shimkets *et al*, 1994). Excess Na^+ passage results from an increased number of Na^+ channels in the cell membrane, due to disruption of a C-terminal protein binding site of the subunit (Schild *et al*, 1995; Snyder *et al*, 1995). The clinical picture of this syndrome relates more to renal impairment than respiratory problems, as it features arterial hypertension, alkalosis and hypokalemia.

2.9.5 Pathologies of liquid clearance

Respiratory morbidity is the most common single cause of hospitalisation in newborn intensive care units (Bohin and Field, 1994). The causes of respiratory disease are diverse but as a matter of opinion it is interesting to consider whether the choice of clinical

nomenclature has influenced growth of understanding.

As very different causes may result in similar or indistinguishable clinical pictures, definitions of neonatal lung disease are mainly based on clinical and radiological signs. An overlap between the clinical descriptive approach, and a patho-physiological description of the respiratory disease, may have contributed to a misleading nomenclature. This is particularly true for the clinical perception related to the term "respiratory distress syndrome", or "RDS", which originally signified a broad and descriptive set of conditions with increased respiratory workload (present in most respiratory pathologies). The suggested cause of RDS now tends to be limited to surfactant deficiency, which may obscure other patho-physiological mechanisms that might contribute to the condition, such as delayed lung liquid clearance.

Transient tachypnea of the newborn or type II respiratory distress syndrome is also known as "wet lung" according to the postulated mechanism underlying the respiratory dysfunction. The definition became restricted to a relatively specific clinical situation involving near-term or term infants (Gomella, 1992). The condition of incomplete lung liquid clearance or "wet lung" is in no way identifiable clinically in the preterm infant, being obscured by other causes of lung disease in prematurity, i.e. particularly surfactant deficiency. Therefore, the term "wet lung" is not used in premature infants, although it may be anticipated that the patho-physiological condition of "wet lung" exists in prematurity, and may even be more common than generally accepted.

"Wet lung"

Term newborn. Respiratory impairment due to insufficient lung liquid clearance in the

near-term newborn has been recognized for many years by clinicians. The first description of 8 cases, all but one born at term by normal vaginal delivery, was published by Avery (Avery *et al*, 1966) who termed the condition "transient tachypnea of the newborn" and speculated on delayed absorption of fluid at birth or "wet lung". In the majority of cases the condition is not severe, manifesting only as mild distress with moderate increase in respiratory workload, typically demonstrated by tachypnea and mild hypoxia requiring minimal treatment and usually less than 40% inspired O_2. Respiratory distress resolves usually within 24 h, which gave the disease its name of "transient tachypnea of the newborn".

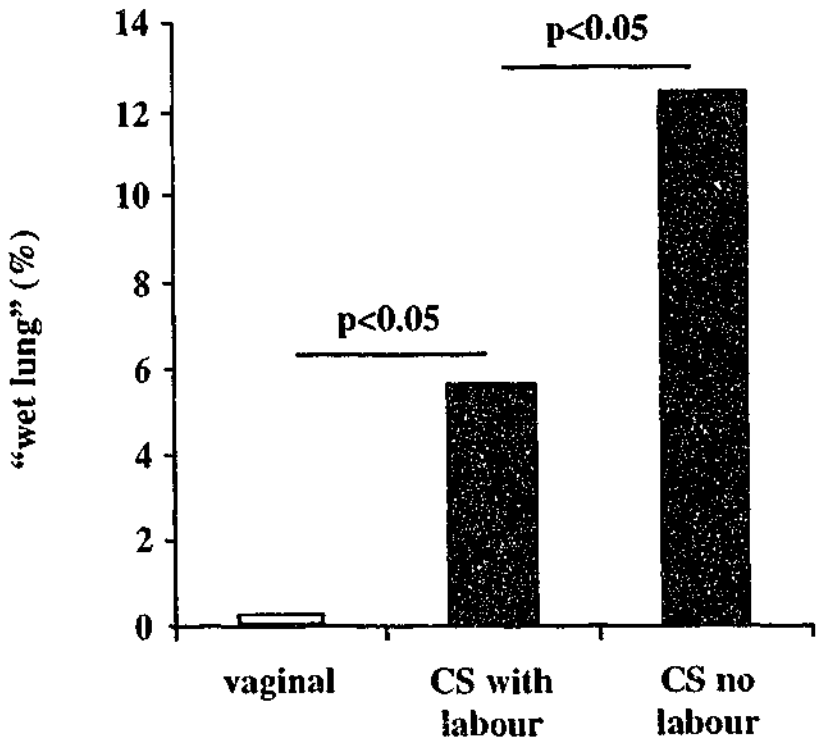

Figure 2-2. *Incidence of "wet lung" in term human infants according to mode of delivery and labour (from Hales et al, 1993).*

Epidemiological data on human birth are consistent and provide an interesting insight into "wet lung", as its incidence, which is below 1% in normal vaginal delivery, increases very significantly after caesarean section (Usher *et al*, 1971; Fedrick and Butler, 1972; Cohen and Carson, 1985; White *et al*, 1985; Krantz *et al*, 1986; Curet *et al*, 1988; Bryan *et al*, 1990). In a group of term babies delivered by caesarean section after labour had begun, the incidence was 5.6% (see Figure 2-2) and this increased to 12.4% when caesarean section was performed without labour (Hales *et al*, 1993). Interestingly, in term infants born after caesarean section and in infants with transient tachypnea of the newborn, the potential difference of the nasal mucosa, which reflects ion transport mechanisms that are active in this epithelium, has been shown to differ significantly from controls (Gowen *et al*, 1988).

Pre-term newborn. Prematurity being very often associated with respiratory distress, the issue of lung maturation has always been a major concern. The early recognition of a strong association between surfactant deficiency and respiratory distress has directed research in this field, but it has also diverted attention from other possible mechanisms, such as delayed lung liquid clearance or "wet lung".

There are several reasons to anticipate that "wet lung" occurs in prematurity, and perhaps more frequently and more severely than in term babies. It seems likely that "wet lung", which has clearly been reported to be more frequent after caesarean section in term babies (Patel *et al*, 1983), would also affect premature babies, since liquid fills the lung of fetuses of comparable post-conceptional ages. Indeed, premature babies born after normal vaginal delivery experience respiratory impairment only half as often as those delivered by caesarean section (see Figure 2-3), where the incidence is further increased in the absence of labour (Robert *et al*, 1976; White *et al*, 1985). In addition, surfactant replacement

therapy, has only reduced but not abolished the morbidity and mortality of the disease in preterm infants, suggesting there is an additional cause of the disease (Jobe, 1993).

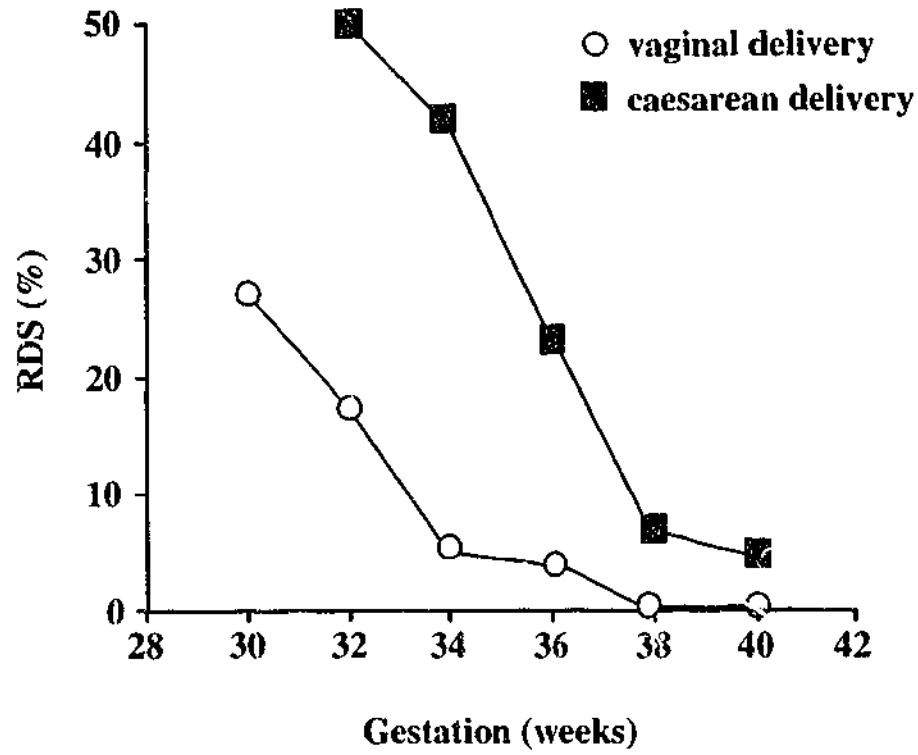

Figure 2-3. *Incidence of RDS in human infants according to delivery mode: vaginally versus caesarean section (from Robert et al, 1976).*

Further clinical evidence that excess lung water is involved in respiratory disease in prematurity comes from the observation that infants dying from RDS have increased lung water content, dilated lung lymphatics and interstitial pulmonary oedema (Lauweryns *et al*, 1968). Interestingly, in the pre-surfactant era, surviving babies with lung immaturity started spontaneous resolution of RDS by the third or fourth day of life, almost invariably preceeded by a increase in urinary output (Langman *et al*, 1981; Spitzer *et al*, 1981; Heaf

et al, 1982) which was called by clinicians "Morgenröte", from the German word for "dawn". Additional strong evidence for increased lung water content in premature infants with RDS is the report of increased signal intensity in magnetic resonance imaging of the lung when compared to premature controls without RDS (Adams *et al*, 2000a; Adams *et al*, 2000b). In those infants with increased water content, distribution was along a gradient with increased water content towards dependent lung zones, a distribution which reversed at least partially after repositioning the infants.

Experimental data also support the hypothesis of deficient liquid clearance being an important factor in premature respiratory morbidity. One of the possible liquid clearing mechanisms, i.e. Na^+ absorption, has been clearly demonstrated to follow a maturational pattern (Kitterman *et al*, 1981a; Brown *et al*, 1983; O'Brodovich *et al*, 1993; Tchepichev *et al*, 1995; O'Brodovich, 1996; Wallace *et al*, 1996b). The potential difference in the pulmonary epithelium mainly reflects the electrogenic transport of the ions Na^+ and Cl^- (Olver *et al*, 1986). Determination of this potential difference in the human alveolar epithelium in vivo is not possible, but it has been suggested that the nasal trans-epithelial potential difference reflects Na^+ and Cl^- transport in the lower airway epithelium (Knowles *et al*, 1982). In the nasal airway the potential difference is clearly reduced in premature human infants with RDS compared to premature infants without RDS (Barker *et al*, 1997), a finding similar to what was observed in term infants with transient tachypnea of the newborn or after caesarean section compared to controls (Gowen *et al*, 1988). Inhibition of the potential difference by amiloride was significantly smaller in RDS than non-RDS group and suggested deficient ENaC activity in RDS infants.

Dry lungs

"Dry lungs" are reported in a very small number of cases (McIntosh, 1988; Losa and Kind, 1998); the definition of "dry lungs" is clinical and requires premature newborns to present with severe respiratory disease at delivery, after prolonged leakage of amniotic fluid had occurred, with very high inflation pressures necessary for ventilation. To differentiate from lung hypoplasia, its main differential diagnosis, a dramatic and rapid improvement has to occur, usually during the first 24 to 36 h, and other respiratory pathologies, in particular infection, have to be excluded. It is hypothesised that this condition is the patho-physiological opposite to "wet lung"; that is, the condition is caused by excessive liquid loss from the lungs. It has been suggested (Losa and Kind, 1998) that the incidence of this condition might be underestimated due to lack of unequivocal clinical presentation. However, the physio-pathological bases for "dry lungs" being a specific disease entity are weak and the cited references fail to provide convincing arguments for it.

2.10 Lung Liquid Clearance in the Perinatal Period

Near the end of gestation liquid fills the airspace to a volume close to functional residual capacity. When air breathing begins without alteration to the volume of this liquid, as is likely after elective caesarean delivery, ventilatory function may be compromised in part because the inflow and outflow of air would occur towards the upper plateau of the pressure-volume curve of the lung. Furthermore, the presence of a large amount of liquid in the parenchyma of the lung would increase the likelihood that airways would become obstructed and thereby adversely affect the ventilation-perfusion ratio of different regions of the lung.

Postnatally, the lung is gas-filled, with a thin film of alveolar or airspace liquid, measuring

in volume less than 1 ml.kg^{-1} body weight (Stephens *et al*, 1996), lining the entire lung epithelium (Untersee *et al*, 1971). This thin layer of liquid has an important influence on the mechanical properties of the lung, providing an elastic recoil that assists expiration. Although the establishment of a relatively "dry" lung may take several hours in normal adaptation after birth, as suggested by clinical experience and experimental work (Bland, 1983; O'Brodovich *et al*, 1990a; Hummler *et al*, 1996; McDonald *et al*, 1999), during adult life the volume remains relatively constant (Stephens *et al*, 1996), except in some diseases of the liquid and ion balance as discussed earlier (Section 2.9).

In recent years it has become clear that a very large decline in lung liquid volume occurs before normal vaginal delivery. Estimates of extravascular lung liquid in labour (Bland *et al*, 1982), and direct determination of lung liquid volume at the end of labour (Berger *et al*, 1998), show that approximately 75% of the liquid occupying the lung in late gestation has already been cleared from the lung before the fetus delivers vaginally. However, the exact timing of this decline, and the mechanisms underlying it, are still under debate. A further issue of importance to pulmonary function is the mechanism by which the remaining 25% of the lung liquid that filled the fetal lung is removed. This issue is of particular interest for caesarean section deliveries where the percentage of liquid remaining in the lung at birth must be much higher. In lambs, lung weight measurements after birth reached a steady state only 4 to 6 hours after birth, suggesting that liquid clearance from the luminal compartment and tissues was completed over this period of time (Humphreys *et al*, 1967; Bland *et al*, 1980).

It is crucial to understand the timing and possible trigger mechanisms that initiate absorption and cause clearance of lung liquid in the perinatal period, since this information

could assist in the development of strategies to prevent neonatal respiratory compromise, in particular the common newborn respiratory disease of "wet lung".

2.10.1 Timing of clearance

One of the physiological effects of the decline in lung liquid volume prior to air-breathing has been demonstrated experimentally in the lamb in our laboratory (Berger *et al*, 1996). Lambs delivered after reduction in lung liquid volume, through aspiration of approximately 50% of the liquid present in the lungs prior caesarean section, showed improved gas exchange when compared with lambs delivered without alteration to their lung liquid volume. However, the lambs in which liquid volume was reduced did not establish gas exchange as effectively as lambs after normal vaginal delivery. This discrepancy in gas exchange could perhaps be accounted for by the fact that at the end of labour the volume of liquid in the air-spaces of the lungs is only 25% of the volume present in the lungs in late gestation (Berger *et al*, 1998), or only half of what remained at the time of caesarean delivery (Berger *et al*, 1996).

Until recent years available evidence supported the view that clearance of liquid from the lung occurs well before delivery, with the process beginning several days before the onset of labour (Dickson *et al*, 1986). Evidence also indicated that lung liquid production falls in the few days preceding labour, as shown by a decline in the flow of liquid out of the trachea (Kitterman *et al*, 1979) and a fall in secretion rate (Dickson *et al*, 1986). By contrast, two reviews which summarise results from one research group (Hooper and Harding, 1995; Harding and Hooper, 1996), together with a new study (Lines *et al*, 1997), contest the findings of a decline in lung liquid volume in late gestation. These reports present data showing that lung liquid volume continues to rise until the onset of labour,

reaching a level as high as 50 ml.kg^{-1} of body weight in fetal sheep. As detailed and discussed later (see Chapter 4), a possible explanation for these contradictory findings lies in the different measurement techniques used. It is striking to note that radio-iodinated serum albumin (RISA) was used in Dickson's study (Dickson *et al*, 1986), whereas Blue Dextran (BD) was used in studies reporting that lung liquid volume continues to rise until labour (Hooper and Harding, 1995; Harding and Hooper, 1996; Lines *et al*, 1997).

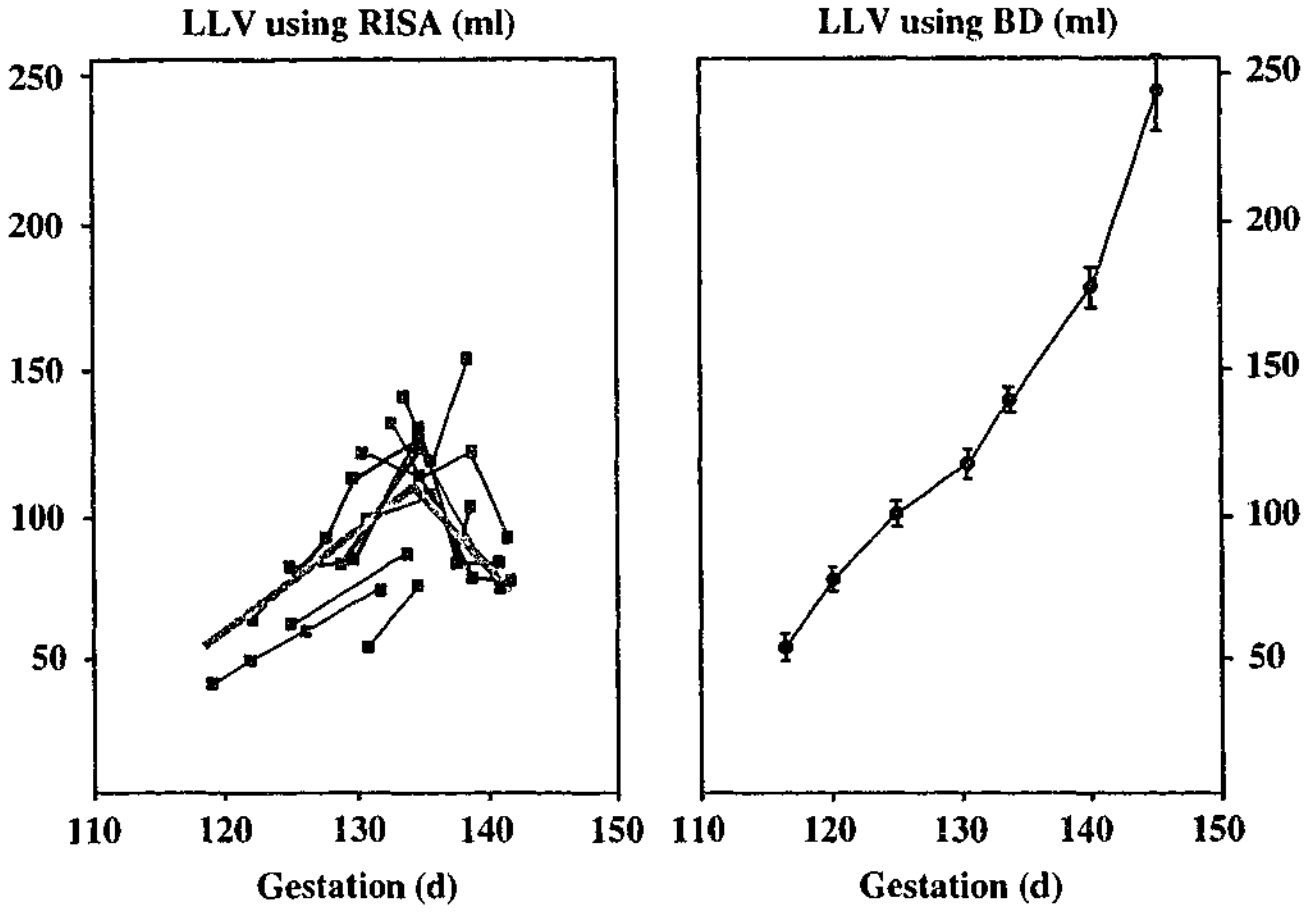

Figure 2-4. *Lung liquid volume in late gestation- the controversy. Note that the left panel shows results from Dickson and co-workers, and the right panel shows results from Harding and co-workers.*

2.10.2 Mechanisms of clearance

Although a number of mechanisms of clearance have been identified, their relative importance in the decline of lung liquid volume in late gestation and labour is at present poorly understood, and the source of some controversy. Conceptually, two pathways of liquid clearance have to be considered, trans-tracheal and trans-epithelial liquid clearance. These mechanisms have been described earlier, but during labour and birth additional driving forces may have to be taken into account.

Trans-tracheal clearance

Passive forces. There is evidence in human pregnancies that amniotic fluid volume falls before term (Nwosu *et al*, 1993; Owen and Ogston, 1996; Salahuddin *et al*, 1998) and it clearly falls after term (Marks and Divon, 1992; Chauhan *et al*, 1999). As discussed earlier, a chronic reduction of amniotic fluid volume results in a fall in lung liquid volume (Dickson and Harding, 1989; Harding and Liggins, 1991), suggesting that loss of amniotic fluid in the lead up to birth could allow compression of the fetus and play a role in clearing liquid from the lung of the fetus. It is also clear that an abrupt loss of amniotic fluid occurs with rupture of membranes during vaginal and caesarean delivery, but whether this loss of amniotic fluid impacts on lung liquid volume has not been examined experimentally. Uterine contractions could also lead to loss of lung liquid, since they provide a powerful force on the fetal chest that would favour expulsion of lung liquid when the fetal nostrils are in the birth canal and increased amniotic fluid pressure can no longer be transmitted to the nostrils to counteract outflow.

During vaginal delivery, compression of the fetal chest as it passes through the narrow birth canal has been postulated to expel liquid by compressing the lungs, a mechanism

called the "vaginal squeeze" (Geubelle *et al*, 1959; Karlberg, 1960; Karlberg *et al*, 1962a). Such a fall in lung liquid volume could be expected to result in negative intra-thoracic pressures immediately after birth, which might explain the reports that in many infants delivered vaginally the first breath appears to occur without inspiratory effort (Karlberg, 1960). Expulsion of liquid from the lung by this method may be facilitated by the high compliance of the fetal thoracic wall which allows a reduction in lung volume well below the level of residual volume in the young animal and adult (Agostoni, 1959). In view of its potential to remove liquid from the lung, and to assist in aeration of the lung, the vaginal squeeze may help to explain the lesser respiratory morbidity of babies delivered vaginally compared with those delivered by caesarean either before or after the start of labour (Robert *et al*, 1976; Hales *et al*, 1993).

Active forces. Any evidence for reduced lung liquid volume and liquid clearance before the fetus is compressed in the birth canal, and before the onset of labour, would clearly point to a fetally-driven mechanism. Although several studies have demonstrated that a decline in lung liquid secretion and volume has occurred when the fetus is in labour (Bland *et al*, 1979; Bland *et al*, 1982; Berger *et al*, 1998), there is at present only limited evidence for a fall in lung liquid volume and secretion rate before the onset of labour (Kitterman *et al*, 1979; Dickson *et al*, 1986).

As discussed in the next section, trans-epithelial absorption of liquid, i.e. across the lung, begins only very close to the end of labour and in the fetal sheep can be estimated to be responsible for clearance of approximately 20 ml of liquid (Brown *et al*, 1983), which corresponds to a maximum of 20% of the volume estimate before onset of labour. Accordingly, some other mechanism must be postulated to account for the 75% decline in

lung liquid volume that has been shown to precede vaginal delivery (Berger *et al*, 1998). Two highly characteristic phenomena that are observed only in labour or the day before (Berger *et al*, 1986) may play an important role in liquid clearance during this period. First of all, fetal breathing movements disappear several days before labour and birth, and experimental interruption of breathing movements has been reported to lead to a reduction in lung liquid volume of about 24% after a period of some days (Miller *et al*, 1993). Second, intermittent "cough like" activity appears during the last days of gestation, characterised by a series of intense inspiratory and expiratory efforts following each other in rapid sequence and generating positive and negative pleural pressures of up to 50 - 80 cm H_2O (Berger *et al*, 1986). Such activity clearly has the potential to expel liquid from the lung, and because the larynx does not allow amniotic fluid to flow back into the lungs it would lead to a decline in lung liquid volume. In addition, tonic activity of the diaphragm and of the muscles of the ribcage and abdomen become prominent during labour (Berger, unpublished observations), and these have the potential to force liquid from the lung.

Trans-epithelial liquid absorption. Although the ENaC clearly plays a role in perinatal lung liquid clearance, as argued earlier, it is unlikely to be the pathway responsible for clearing the major part of the liquid removed from the lung before birth, because it is activated only very close to the end of labour. While removal of a small volume of liquid from the lung just before delivery may have a pronounced impact on postnatal gas exchange (Berger *et al*, 1996), the major role of the ENaC may not occur until after birth. The ENaC at this time may first serve to remove residual liquid from the lung that had not been cleared prenatally, and then ensure that the lung is kept "dry" in the face of any water leakage into the alveolar space (Goodman *et al*, 1987; Matthay *et al*, 1998).

While prenatally the ENaC is activated by adrenaline and other agents that rise during labour, some redundancy in the mechanisms activating absorption is likely in view of the crucial importance of this mechanism, as indicated by the fact that mice without functional ENaC die soon after birth of respiratory failure. Death in these animals was associated with an excess of liquid in the lung (Hummler *et al*, 1996) and it clearly points to absorption of liquid from the lung as a crucial component in the adaptation of the fetus for postnatal life. A mechanism that appeals as a potential second trigger for activating the ENaC is lung expansion, since expansion is known to increase absorption in the rabbit bladder (Lewis and de Moura, 1982) and frog skin (Nutbourne, 1968), and lung expansion is the norm after birth. Support for this possible trigger comes from experiments on the artificially-perfused postnatal lung of the lamb in which expansion was shown to increase the rate of liquid reabsorption from the airspace by stimulating active transport of Na^+ across the epithelium (Ramsden and Neil, 1993). The existence of a similar mechanism of activation of Na^+ transport in the fetus has not been demonstrated as yet, although it has been shown in the fetal goat that artificially increasing lung liquid volume may result in a decline in liquid secretion, and, in some cases, liquid absorption (Perks and Cassin, 1985b). Understanding this mechanism may offer a new insight into the as yet unexplained "switch" in the lung at birth from an organ which secretes to one that absorbs liquid, even though the levels of labour-associated triggers of the ENaC have returned to normal. Such a mechanism may have important clinical implications, explaining for example why CPAP and PEEP (which also distend the lung) have such a beneficial effect upon gas exchange in many conditions in which the lung is "wet", including transient tachypnea of the newborn, RDS in the preterm infant and a number of adult pulmonary diseases (Matthay, 1985).

CHAPTER THREE

EXPERIMENTAL OBJECTIVES

EXPERIMENTAL OBJECTIVES

3.1 Major Questions

Although lung liquid secretion is essential for normal fetal development, both experimental (Berger *et al*, 1996) and clinical (Hales *et al*, 1993; Morrison *et al*, 1995) evidence unequivocally supports the intuitive view that lung liquid must be cleared for effective gas exchange after birth. It has recently been shown that the bulk of this liquid leaves the lungs before birth (Berger *et al*, 1998), but the exact timing of this prenatal liquid clearance has not yet been resolved and remains a major question. Some relatively recent reports suggest that lung liquid volume continues to rise until the day before labour, reaching very high levels up to 50 $ml.kg^{-1}$ of body weight (Hooper and Harding, 1995; Harding and Hooper, 1996; Lines *et al*, 1997) contradicting the only available published evidence until then that lung liquid volume begins to decline several days before labour (Dickson *et al*, 1986).

A possible explanation for the contradictory findings about the volume of liquid present in the lungs of the late-gestation fetal sheep suggested itself when it became evident that differing results originated from research groups using different measurement techniques. In most studies of fetal lung liquid, volume is determined using indicator dilution. In the study reporting that lung liquid volume declines before labour, the indicator or tracer used was radio-iodinated serum albumin (RISA) whereas Blue Dextran (BD) was used in studies reporting that lung liquid volume continues to rise until labour. As the dye moiety of BD is known for its high affinity for proteins (Dean and Watson, 1979), we suspected that BD may bind to cell-surface proteins on the apical surface of the maturing lung epithelium such binding would contravene a fundamental requirement of the indicator-dilution technique that the tracer should remain freely dispersed and available, and it would clearly give rise to an over-estimate of lung liquid volume.

The first question addressed in this Thesis was therefore whether BD is a reliable tracer for lung liquid volume determinations. A particular concern with the use of BD as a lung volume tracer was that binding to epithelial cells in the lung lumen may change with gestation, as the epithelial cells could be expected to increase surface glyco-proteins and therefore alter protein density with advancing gestation. Thus, it was conceivable that the rise in lung liquid volume reported in studies using BD could be an artefact, and that volume may actually have fallen. On the other hand, the study in which a decline in lung liquid volume was reported before labour (Dickson *et al*, 1986) is also subject to criticism, in that labour was not routinely monitored and diagnosed. Thus, some of the animals studied may have been in early labour at the time a decline in lung liquid volume was observed. On the basis of existing work it is evident that considerable uncertainty remains as to the simple question of the timing of water clearance in the days leading up to labour.

The second goal of this Thesis was hence to determine the time course with which lung liquid volume declines in the last week of gestation, with specific focus on whether absorption of liquid across the pulmonary epithelium plays a major role in the clearance of liquid. To unequivocally solve the debate we used RISA as the volume tracer, since our first series of experiments had validated its use in the late gestation fetal lung.

Finally, for air-breathing the lung must be permanently transformed from a secretory organ to one which is characterised by liquid absorption, raising the third question addressed in this Thesis; what triggers this vital transformation of the pulmonary epithelium? As discussed in detail earlier, the transformation from secretion to absorption at birth may simply be achieved by shifting from Cl^- secretion to Na^+ absorption (Olver *et al*, 1986). Although adrenaline is capable of initiating absorption near the end of labour (Brown *et al*,

1983), its circulating level falls rapidly after delivery. Until now, however, the mechanism that maintains the lung in a permanent absorptive state after birth remains unclear. A stimulus that continues throughout postnatal life is particularly appealing, and one such stimulus is lung stretch caused by air breathing itself. Some evidence is indeed available that stretching of epithelial cells may activate Na^+/K^+-ATPase (Waters *et al*, 1999) and that lung liquid is absorbed in response to expansion of the lung (Egan, 1976; Perks and Cassin, 1985b; Ramsden and Neil, 1993; Vejlstrup *et al*, 1994; Kojwang and Perks, 1996). The possibility that lung stretch is a trigger for transforming the lung at birth into an absorptive organ is addressed in the Thesis using for the first time an intact late gestation fetal sheep model in vivo.

3.2 Experimental Program

As the review of published data on the timing of liquid clearance suggested a possible relationship between the results obtained and the tracer used, preliminary experiments for this Thesis were undertaken with the two tracers in question, BD and RISA. In this setting, it became clear that the two tracers, which were supposed to be equivalent, gave discrepant results and reinforced the doubts that arose in the literature review. An in-depth evaluation of the techniques appeared therefore essential. Interestingly, despite extensive previous use over many years, several aspects of both volume tracers lacked a detailed validation. Thus, rigorous testing of the tracer dilution techniques currently in use was considered essential, on the one hand to validate a tool for measurements in later experiments, and on the other hand to suggest an explanation for some of the controversies in the literature. To achieve this aim, the first step was to evaluate whether one or both of the commonly used tracers, RISA and BD, satisfy the assumptions underlying the indicator dilution technique, and to quantify the extent to which they contravene these assumptions (Chapter 4).

After assessing the accuracy of the techniques upon which the current literature in this field is based, and validating a tool for further experiments, the Thesis then aimed to establish the timing, possible triggers and mechanisms that lead to a decline in lung liquid volume, cessation of lung liquid secretion and the initiation of absorption in the perinatal period. This information is essential for developing strategies to prevent neonatal respiratory compromise, one of the most common neonatal morbidities. As the bulk of studies on the timing of perinatal lung liquid clearance based on serial or longitudinal determinations of lung liquid volume may be criticised because of major technical flaws, in Chapter 5 we re-explore the natural time course of fetal lung liquid volume longitudinally, with a particular focus on late gestation and labour, using the validated RISA technique. Furthermore, our studies sought to explore the mechanisms by which any change in lung liquid volume occurs, in particular whether decline in secretion and initiation of absorption across the pulmonary epithelium play a role.

Finally, in Chapter 6, the possibility that stretch induces lung liquid absorption is explored, by measuring lung liquid secretion rate while expanding the fetal lung with liquid. While there have been reports in particular animal preparations suggesting this effect occurs, no complete study had been performed in vivo under physiological conditions equivalent to those in our chronic instrumented fetal sheep model.

3.3 Choice of an Animal Model

As lung liquid is present during fetal life in all the mammals that have been studied, the underlying supposition for this work is that the maturation pathways and function of lung liquid are similar in all species. Thus, the findings of this Thesis may be assumed to apply equally to the human fetus and newborn, and are likely to provide a basis for

understanding respiratory pathology in the newborn infant.

The sheep has been the most popular species used for lung liquid studies, probably because of its large size which makes it suitable for surgical instrumentation and physiological studies, and for its ready availability. Many studies have successfully modelled multiple pathologies of human lung development such as lung immaturity, diaphragmatic hernia, lung hypoplasia using the fetal, premature or neonatal sheep and confirmed the adequacy of the model for those questions.

3.4 Generation of Hypotheses

The following basic hypotheses and specific working hypotheses were examined and represent the foundations of this Thesis:

3.4.1 Hypothesis 1a: BD contravenes the requirements for an impermeant tracer

BD binds to the pulmonary epithelium in an age-dependent fashion, thereby contravening the assumptions of the impermeant tracer method for determining fetal lung liquid volume.

3.4.2 Hypothesis 1b: RISA satisfies the requirements for an impermeant tracer

RISA satisfies the assumptions of the indicator dilution technique in the late gestation fetal sheep, by remaining freely available and dispersed within the liquid filling the lung lumen thereby allowing easy and precise determination of its concentration.

3.4.3 Hypothesis 2: V_L and J_v fall during late gestation and labour

At the end of gestation, and before the onset of labour, lung liquid volume begins to diminish and the rate of secretion of lung liquid declines.

3.4.4 Hypothesis 3: Lung expansion triggers active lung liquid clearance

In the late gestation fetal sheep in vivo, cyclic lung expansion that mimics volume changes associated with postnatal respiration causes reduced lung liquid secretion or induces absorption by triggering the activation of an amiloride-sensitive Na^+ transport mechanism.

CHAPTER FOUR

COMPARISON OF RADIO-IODINATED ALBUMIN AND BLUE DEXTRAN FOR ESTIMATING LUNG LIQUID VOLUME IN FETAL SHEEP

COMPARISON OF RADIO-IODINATED SERUM ALBUMIN AND BLUE DEXTRAN FOR ESTIMATING LUNG LIQUID VOLUME IN FETAL SHEEP

4.1 ABSTRACT

Fetal lung liquid volume is usually determined using radio-iodinated serum albumin (RISA) or Blue Dextran (BD) as volume tracers. We tested the reliability of both tracers at 124 and 142 days gestation (G124 and G142; term = G147) when the labels were employed simultaneously. We measured the proportion of label bound reversibly to the lung, or apparently lost from the lung compartment, by washing out the lung with saline and 5% albumin. At G124, volume estimates with the two labels were similar. At G142, the volume estimate with BD (36.3 ± 8.7 $ml.kg^{-1}$ of body weight) was higher ($p < 0.05$) than with RISA (22.3 ± 3.5 $ml.kg^{-1}$). This difference resulted from reversible binding of BD, since 5% albumin wash-out released 38.5 ± 4.0% of the BD added at the start of the experiment, but a lesser amount of RISA (9.8 ± 0.7%: $p < 0.05$). At G142, when RISA was used alone its reversible binding was 1.3 ± 0.2%. Background absorbance increased during experiments, giving rise to an apparent increase in BD concentration. We conclude that RISA is an effective tracer for lung liquid volume determination in the fetal sheep, whereas our findings of substantial epithelial binding of BD, and large changes in background absorbance, demonstrate that under the conditions of our experiments BD is a poor tracer close to term.

4.2 INTRODUCTION

The fetal lung secretes a liquid which distends the future airspace and plays a crucial role in promoting lung growth (Alcorn *et al*, 1977). While lung liquid plays an important role in

fetal development, it must be cleared from the lungs for effective gas exchange after birth. Recent evidence demonstrates that the bulk of this liquid leaves the lungs before birth (Berger *et al*, 1998) but the exact timing of this prenatal clearance is in dispute. Until recently the only available published evidence suggested that lung liquid volume begins to decline several days before labour (Dickson *et al*, 1986). However, a number of recent reports suggest that lung liquid volume continues to rise until the day before labour, reaching a level as high as 50 $ml.kg^{-1}$ of body weight (Hooper and Harding, 1995; Harding and Hooper, 1996; Lines *et al*, 1997). A possible explanation for the contradictory findings about the volume of liquid present in the lungs of the late-gestation fetal sheep is that they result from differences in measurement techniques. In most studies of fetal lung liquid, its volume is determined by the indicator dilution technique in which a known amount of tracer is completely mixed into the liquid occupying the airways and alveoli of the lung compartment. Volume is then determined by dividing the quantity of tracer added by its concentration in lung liquid. In the study reporting that lung liquid volume declines before labour (Dickson *et al*, 1986), the indicator or tracer used was radio-iodinated serum albumin (RISA) whereas Blue Dextran (BD) was used in studies reporting that lung liquid volume continues to rise until labour (Hooper and Harding, 1995; Harding and Hooper, 1996; Lines *et al*, 1997).

The dye moiety of BD (Cibacron Blue F3-GA) is known to have a high affinity for proteins (Dean and Watson, 1979) a property that has led to its frequent use in protein separation techniques (Gianazza and Arnaud, 1982; Sakoda *et al*, 1990; Horvath *et al*, 1996). Accordingly, we suspected that BD may bind to cell-surface proteins on the apical surface of the maturing lung epithelium, just as it binds to the pulmonary vascular endothelium (Nelin *et al*, 1992; Roerig *et al*, 1992). If this were so, BD would fail to

satisfy a fundamental requirement of the indicator-dilution technique that the tracer should remain freely dispersed. As a result, use of BD would give rise to an over-estimate of lung liquid volume. We therefore set up a study in which we evaluated BD and RISA in the measurement of lung liquid volume at a gestational age at which lung liquid volumes determined with the two tracers are similar (G124), and at a gestational age at which reported volumes are widely different (G142). We were especially interested in how much of each tracer remained freely available during experimental conditions in vivo. Whereas most tracer validation studies have searched for tracer "lost" from the compartment of interest, in our novel approach we focused particularly on the amount of tracer that could be retrieved from the lung compartment itself, since it is the freely available or retrievable tracer that is the basis for the volume calculation.

4.3 Materials and Methods

4.3.1 Surgery

Ethical approval was obtained from the Monash University Standing Committee on Ethics in Animal Experimentation for all in vivo experiments.

Twenty fetuses of pregnant Border-Leicester ewes were instrumented at G120 or G133 (term = G147) for study at G124 or G142. A further 3 fetuses were instrumented on the day of an acute study, either at G141 or G144. In all operations, anaesthesia was induced with iv thiopental sodium (1-2 g: Pentothal, Abbott, Australia) and maintained with 66% N_2O: 1-2% Halothane: 32-33% O_2. The maternal abdomen was opened in the midline and the fetal head was delivered through a uterine incision and the neck was opened in the ventral midline. The carotid artery was catheterised non-occlusively using a Teflon, cannula-Tygon, tubing assembly. Two wide-bore Silastic catheters (ID 3 mm, OD 6 mm;

Sil-Med Corporation, Taunton, Massachusetts) were introduced through a single tracheostomy, with one catheter directed rostrally and the other caudally and the ends connected to form a liquid-filled loop. A liquid-filled catheter open to the amniotic space was secured to the skin of the fetal neck. The fetus was returned to the uterus which was carefully closed to avoid leakage of amniotic fluid. The ewe's abdominal wall was sutured and all catheters tunnelled subcutaneously to the animal's flank where they exited through a small incision. All animals received post-operative analgesia (50 mg Finadyne, Schering-Plough, Australia) and daily intramuscular antibiotic treatment (500 mg procaine penicillin, 500 mg dihydrostreptomycin). Animals were allowed to recover for 4 days or more before observations commenced.

4.3.2 Preparation of tracers

RISA

Radio-labelled serum albumin (RISA) was prepared by iodination (I^{125}) of bovine serum albumin (BSA) using Iodo-Gen® (Pierce, Rockford) by the technique of Fraker and Speck (Fraker and Speck, 1978) no more than 6 weeks before use. To remove free I^{125} the tracer was either mixed with Amberlite IRA-400 (Sigma, USA) and allowed to stand for 15 min immediately before use, or it was passed over a Sephadex® G-25 column (Column PD-10, Pharmacia Biotech, Sweden). Specific activity of RISA approximated 20 $\mu C.ml^{-1}$ and the albumin concentration was ~ 0.5%. In each experiment, 0.1-0.2 ml of stock solution was diluted with 3-5 ml of saline (0.9%) to which fetal serum (0.5-1 ml) was added. As a result, the concentration of unlabelled or "cold" albumin exceeded that of RISA by at least an order of magnitude, with the aim of limiting any loss of label that would result from albumin binding to the lung. The tracer was then passed through a millipore filter (0.22µm Millex®-GV filter unit, Millipore, MA) to ensure sterility and to exclude Amberlite from

the sample.

BD

A solution of Blue Dextran was obtained by dissolving dry BD powder (Sigma, Australia) in lung liquid maintained at 40°C. Complete dissolution was achieved by continuous stirring for 15 min.

4.3.3 Measurement of tracer concentration

RISA

Concentration of RISA was measured on a gamma counter (1282 Compugamma, LKB, Wallac) in 0.5 ml samples counted for 10 min on 3 successive runs through a counting window set between 20 and 100 KeV, and the average of the three runs was used in calculations.

BD

Concentration of BD was determined in 1 ml samples by measuring absorbance at the standard wavelength of 620 nm (Perks and Cassin, 1985b; Cassin *et al*, 1994; Lines *et al*, 1997) in a spectrophotometer (Ultraspec III, Pharmacia LKB). Samples were analysed before and after centrifugation at 2000 x g for 60 min.

4.3.4 Calculation of liquid secretion rate and volume

Lung liquid volume was calculated according to established techniques (Brown *et al*, 1983). Slopes of lung liquid volume against time were calculated using the least squares method, after removing values obtained during the first 20 min of the experiment, since these may be affected by incomplete mixing. Lung liquid volume at the time of

interruption of the tracheal loop (time zero) was obtained by extrapolation.

4.3.5 Physical and chemical properties of BD in vitro

The time taken for dissolution of BD was established by adding BD (2 $mg.ml^{-1}$) to a sample of water or lung liquid which was maintained at 40°C and stirred continuously. After addition of BD, samples were taken from the mixture at 5, 10, 15, 30 and 60 min, as well as 24 h later. After 15 min, absorbance had reached more than 97% of the value determined at 24 h, and this time was independent of the solvent. As proof of the complete dissolution of BD at the higher concentrations used in vivo (see below), we sought to spin out undissolved BD at a concentration of up to 6 $mg.ml^{-1}$ after 15, 30, 45 and 60 min of stirring. Absorbance levels before and after spinning for 60 min at 2000 x g were not different for any duration of stirring.

BD absorbance was directly proportional to its concentration between 0 and 3 $mg.ml^{-1}$ and independent of solvent, the slope of the relationship between absorbance and BD concentration being similar for H_2O, 0.9% NaCl and 5% bovine serum albumin (5% BSA). By contrast, the absorbance of BD was reduced by 21.7% when amiloride hydrochloride (Amiloride, Sigma, USA) was present at a concentration of 10^{-4} M in water. BD absorbance was not affected when incubation temperature was reduced from 40°C to 4°C. Nor was the absorbance of BD altered if samples were stored in darkness or exposed for different time periods (1-180 min) to strong artificial light to mimic laboratory conditions.

The possibility that BD might sediment or spin out was assessed in samples of BD (0-2.5 $mg.ml^{-1}$) diluted either in normal saline (n = 13) or 5% BSA (n = 13). Centrifugation at 2000 x g for different time periods up to a maximum of 60 min did not change absorbance

of BD in saline or in lung liquid (see Section 4.4.3). However, when BD in 5% BSA was spun, we found a small but significant 7% reduction of absorbance after 60 min at 2000 x g. This small reduction in absorbance in 5% BSA would have no effect on the magnitude of what we call the "free" fraction of BD (see Section 4.4.1), but it would cause the "bound" fraction of BD to be slightly underestimated, and the "irrecoverable" fraction of BD to be correspondingly overestimated.

4.3.6 Characteristics of BD and RISA in vivo

We employed four experimental protocols. The first ("BD+RISA") aimed to compare BD and RISA as lung liquid volume tracers in studies utilizing both tracers either at G124 (n = 5) or G142 (n = 7). The second ("RISA-only") investigated the accuracy of RISA when used alone in fetuses at G142 (n = 7). The third protocol ("BD-only"), in which BD was used alone at G142, was performed on a single animal to check whether the high binding of BD to the lung, as demonstrated in the "BD+RISA" protocol, was dependent upon the presence of RISA. These three protocols consisted of the same four experimental periods, each lasting approximately 90 min. Lung liquid volume and secretion rate were determined with BD over the first period. After adding RISA, volume and secretion rate were determined simultaneously with both tracers during the second period, which was followed by two wash-out periods, the first with saline, the second with 5% BSA. A fourth protocol ("BD+RISA: acute preparation") was performed in order to address the objection that the high binding of BD we observed in the lung in late gestation results from infection giving rise to an abnormal increase in epithelial cell surface proteins, or to an increase in the particulate content of lung liquid. Accordingly, we performed acute studies in 3 late-gestation fetuses immediately after completion of sterile surgery.

Protocol 1 "BD+RISA": comparison of BD and RISA in simultaneous use

Experimental Period 1: addition of BD. The limb of the tracheal loop directed towards the lung was connected to a temperature-controlled (40°C) glass burette closed at its top by a rubber stopper penetrated by an 18-gauge needle to which a millipore filter was attached. This allowed liquid to be drained from, and re-instilled into, the lung under sterile conditions while minimizing evaporative water loss. A volume of 50-100 ml of liquid was obtained from the lungs and a small sample was taken to measure the background absorbance and radioactivity of lung liquid. BD (250-300 mg) was then mixed by stirring for 15 min into a sample of lung liquid (50-100 ml) and a specimen (3 ml) was withheld before the remaining liquid was accurately weighed (Mettler AE 166 delta range) and re-instilled into the lung. The tracer was mixed with lung liquid by repeated cycles of drainage and re-instillation over at least 30 min using a maximum hydrostatic pressure of ± 15 cm H_2O. Subsequently, between 12 and 15 samples (1 ml) were taken at 5-10 min intervals and absorbance at 620 nm was measured.

Experimental Period 2: addition of RISA. After draining lung liquid into the burette we again withdrew 50-100 ml of this liquid, now containing BD, to which we added RISA. After thorough mixing a 1.5 ml sample was taken to determine radioactivity level, and the volume of liquid remaining was accurately weighed before it was re-instilled and mixed into the lung over a 30 min period as described above. A further 12-15 samples (each 1.5 ml) were taken at 5-10 min intervals for determination of absorbance and gamma radiation.

Experimental Period 3: saline washout. We then sought to retrieve both tracers from the lung by repeated wash-out. The procedure entailed draining as much liquid as possible from the lungs and replacing it with approximately 20 $ml.kg^{-1}$ of isotonic saline at 40°C.

This was repeated 7 times, and resulted at the final wash in BD and RISA levels close to background.

Experimental Period 4: albumin washout. Following the saline washes we performed another series of 7 washes with 5% BSA. Albumin has a very high affinity for BD (Travis and Pannell, 1973; Angal and Dean, 1977; Dean and Watson, 1979; Gianazza and Arnaud, 1982) and we expected it would release any tracer bound to the epithelium, just as it releases BD from the pulmonary endothelium (Nelin *et al*, 1992; Roerig *et al*, 1992). We further detailed whether either volume tracer had crossed the pulmonary epithelium by sampling fetal carotid artery blood (n = 5) before and 5, 10, 20, 30, 60, 120 and 240 min after introducing the tracer to the lung. At the end of the experiment all animals were sacrificed and maternal blood and the following fetal and maternal organ samples were analysed for the presence of tracer: lung, thyroid, liver, kidney, heart, muscle and skin.

Protocol 2. "RISA-only": evaluation of RISA used alone as a volume tracer

This protocol, performed only at G142 (n = 7), incorporated all four steps described in the previous protocol, except that no BD was used. During experimental Period 1, no tracer was present and during experimental Period 2 RISA was added. This experiment provided an estimate of the free, bound and irrecoverable tracer that could be compared with the values obtained in the "BD+RISA" protocol. Any adverse effect of BD on RISA as a volume tracer, and especially whether the presence of BD altered the amount of bound and irrecoverable RISA, would be revealed by the difference in results obtained between the two protocols.

Background absorbance. In preliminary experiments employing RISA alone, the initially

clear lung liquid draining into the burette appeared to become more opaque during the course of the experiment. To establish whether this change in opacity increases background absorbance, which would falsely elevate the BD concentration of the liquid, we measured absorbance at 620 nm in every third sample in Periods 1 and 2, and in every sample of Periods 3 and 4 during the "RISA-only" protocol. To examine whether particulate components in lung liquid may cause this effect, we then centrifuged the same samples for 60 min at 2000 x g before measuring absorbance again.

To examine whether BD binds to the material responsible for increasing the opacity of lung liquid, BD was added to selected samples and absorbance measured before and after centrifugation at 2000 x g. For this purpose we chose the 3rd sample of lung liquid (n = 7) taken in Period 1 of the "RISA-only" protocol, since this sample had a high background absorbance. Absorbance of the sample was measured before and after centrifugation. Then an amount of BD was added to each sample to give a concentration ranging between 0.5-2.5 $mg.ml^{-1}$, mixed thoroughly and absorbance measured again. The samples were then centrifuged as above and absorbance remeasured. If BD were to bind to this material then we would predict that the change in absorbance on centrifugation would be greater in the presence of BD than in its absence.

Protocol 3. "BD-only": evaluation of BD used alone as a volume tracer

In this protocol all four steps described in protocols 1 and 2 were performed in a single animal at G142, except that no RISA was used. This experiment was performed to test whether the presence of RISA affects the amount of BD that is bound to the lung or is irrecoverable by washout.

Protocol 4. "BD+RISA": acute preparation

This experiment was designed to evaluate the possibility that infection introduced at the time of surgery in protocols 1 and 3 caused a response in the lungs over ensuing days that led to a high level of BD binding. Surgery was performed as in the earlier protocols under sterile conditions in 3 fetuses (2 at G141 and 1 at G144) and the volume determination was performed immediately afterwards. The protocol used here began at experimental Period 2, as described earlier. BD and RISA were added simultaneously to approximately 50 ml of lung liquid, and samples were taken to provide an estimate of lung liquid volume with both tracers. At the end of the volume determination, all lung liquid was returned to the lung, the trachea was clamped, the ewe and fetus were killed and the lung removed and weighed. Seven saline washes were then performed, followed by 7 washes with 5% albumin.

Samples of each wash were taken for determination of absorbance and radioactivity. The lungs were dried to constant weight over a period of approximately 2 weeks and the weight was expressed as $g.kg^{-1}$ body weight. Using the factor scaling dry lung weight to normal wet parenchymal weight (5.29) in the newborn lung (Berger *et al*, 1998), we could estimate lung tissue weight and obtain the volume of liquid in the airspace by subtracting lung tissue weight from total wet weight at post-mortem. This value represents the volume of liquid in the lung at the time the fetus was killed, and could be compared with the volume estimated with BD and with RISA at the same time.

4.3.7 Data analysis and presentation

Raw tracer concentration and volume of samples were used to calculate the amount of tracer removed with each sample, and knowing the total amount added we calculated the pool of tracer remaining in the fetus at each step of the protocol. This value was expressed

as a fraction of the pool introduced at the start of the experiment. For Figure 4-4, we recalculated lung liquid volumes taking into account our finding that background absorbance changes during an experiment; for this calculation we used the absorbance of centrifuged samples. In addition, we recalculated lung liquid volume using only the fraction of the BD that is freely available in the lung compartment. Comparisons between groups were performed using Student's paired and unpaired t-tests for normally-distributed data, otherwise by the Wilcoxon signed ranks test. ANOVA was used to compare the lung liquid volume estimates derived in the 3 acute studies. Results are expressed as mean ± SE unless otherwise stated. Differences were considered significant when $p < 0.05$.

4.4 Results

We conducted experiments on 20 chronically-instrumented healthy fetuses, as indicated by their weights, arterial blood gases and acid-base status (Table 4-1), and post-mortem examination.

Table 4-1. Blood gas and pH values of the fetuses in the three study groups

	BD+RISA		RISA only	BD only
n	5	7	7	1
GA, days	124	142	142	142
Weight, kg	3.1 ± 0.2	5.0 ± 0.5	4.4 ± 0.3	5.4
pHa	7.37 ± 0.01	7.33 ± 0.02	7.35 ± 0.01	7.35
PaO_2, mmHg	22.2 ± 1.0	23.6 ± 0.7	23.2 ± 1.7	26.7
$PaCO_2$, mmHg	54.7 ± 1.0	54.1 ± 0.7	51.7 ± 1.7	50.3

Values are given as mean ± SEM. Number of fetuses in each group is given in the upper row.

A further 3 fetuses were studied immediately after acute instrumentation.

4.4.1 Tracer availability

The upper panels of Figure 4-1 (see next page) show tracer concentration (absorbance or $CPM.ml^{-1}$) in each sample of lung liquid plotted against time. Secretion of lung liquid caused tracer concentration to fall gradually in experimental Periods 1 and 2 of the experiment. In Period 3, successive washes of the lung with saline resulted in a rapid and exponential fall of tracer concentration to a value close to zero. In Period 4, addition of 5% BSA resulted initially in a substantial increase in BD concentration, and a minimal increase of RISA. Subsequent BSA washes led to the concentration of both tracers falling exponentially towards zero.

In the lower panels of Figure 4-1, the decline in each tracer pool remaining in the fetus is plotted against time. Removal of samples during Periods 1 and 2 slowly reduced the tracer pool size, until a substantial fall in the pool occurred after the last sample in Period 2 when we removed as much liquid as possible from the lungs. Saline washes in Period 3 exponentially decreased the pool to a first plateau level.

A second plateau was reached with the 5% BSA wash-out in Period 4. The fraction of the pool that lies above the first plateau represents a freely available fraction that is readily retrieved by sampling and saline wash-out ("free fraction"), whereas the fraction between the first and second plateau represents a "bound fraction" that is unavailable until released by 5% BSA wash-out. The tracer pool that lies below the second plateau could not be recovered from the lung compartment ("irrecoverable fraction").

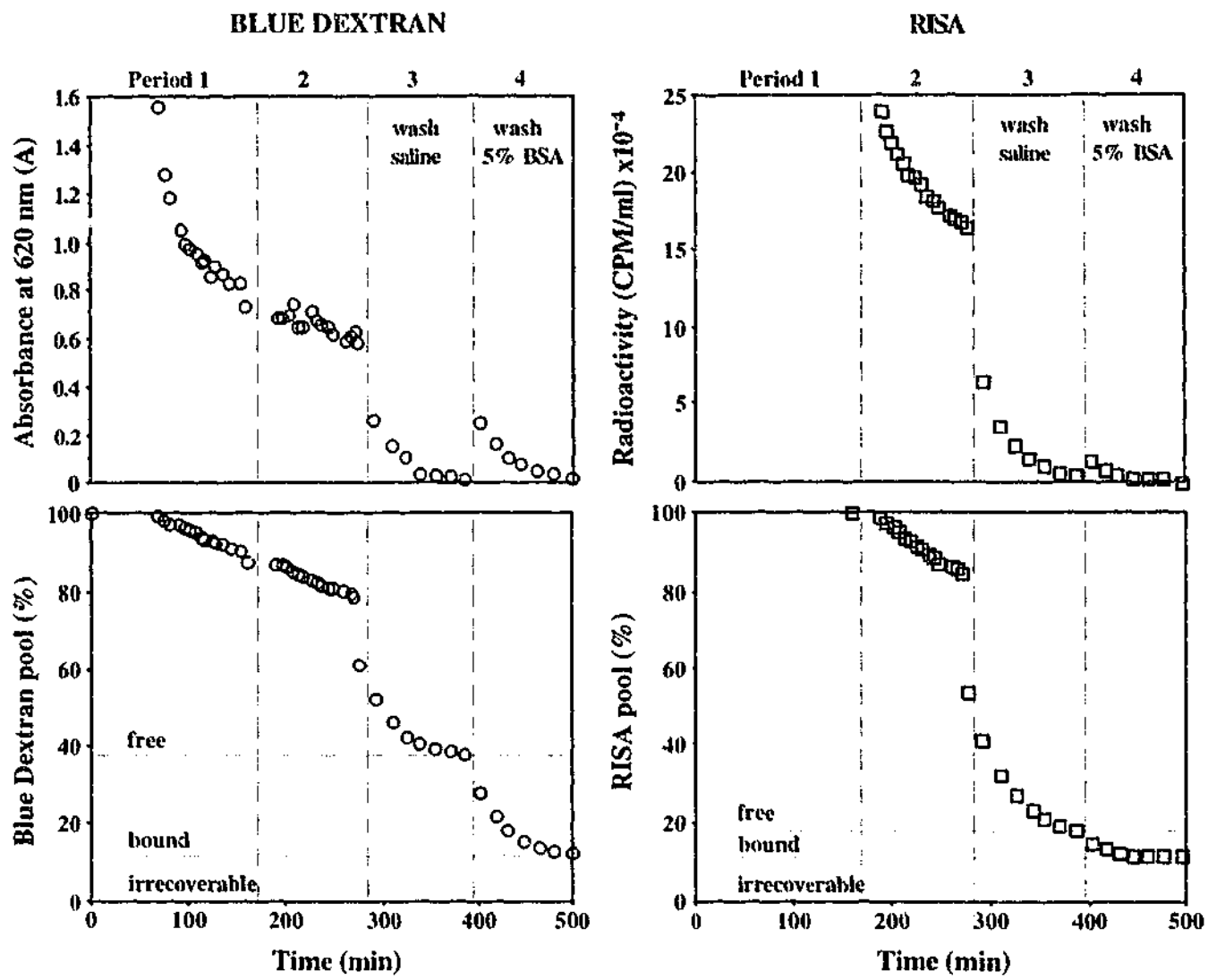

Figure 4-1. *Typical experiment of the "BD+RISA" protocol at G142, showing tracer concentration (upper panels) and pool size (lower panels) levels plotted against time. Results obtained with BD are shown on the left and with RISA on the right. During the first two experimental periods, concentrations of BD and RISA decreased due to lung liquid secretion and tracer removed with sampling. The initial exponential fall in concentration indicates that steady state (mixing or binding) was not achieved in these first samples. The horizontal dashed lines indicate the fractions referred to as "free", "bound" and "irrecoverable".*

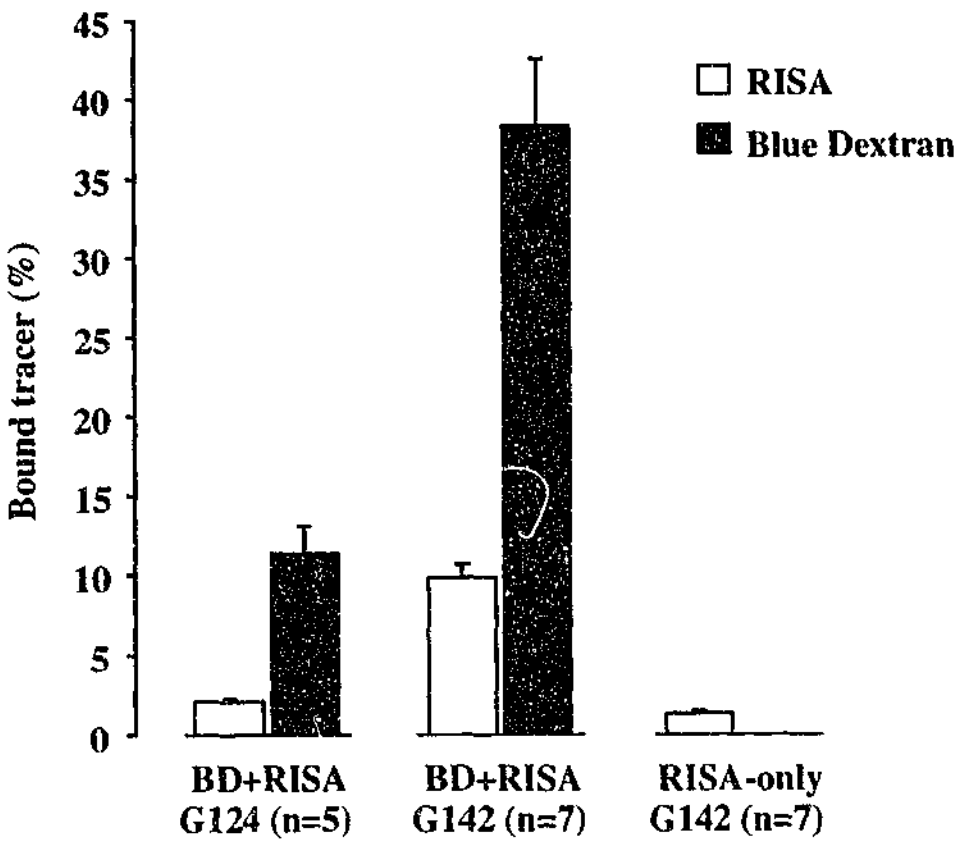

***Figure 4-2**. Bound BD and RISA at G124 and G142 in the "BD+ RISA" protocol and in the "RISA-only" protocol.*

Average results for the 7 experiments are illustrated in Figure 4-2. The fraction of both BD and RISA that was bound was 4-5 fold greater at G142 than at G124. At both gestations, significantly more BD than RISA was bound to the pulmonary epithelium: thus at G124, 11.3 ± 1.9% of BD was bound compared with 2.2 ± 0.04% of RISA ($p < 0.05$), while at G142, 38.5 ± 4.0% of BD was bound compared with 9.8 ± 0.7% of RISA ($p < 0.05$). When RISA was used alone, its bound fraction was significantly smaller (see below: "Effect of BD on RISA binding").

In the "BD+RISA" protocol, at G124 the irrecoverable BD (2.9 ± 3.3%) was not different from the value at G142 (5.1 ± 3.8%); nor was the irrecoverable fraction of RISA different between G124 (4.8 ± 0.3%) and G142 (8.2 ± 1.6%). In the "RISA-only" protocol, 4.9 ± 0.8% of the added RISA was irrecoverable, a value that was not significantly different from the irrecoverable RISA at G142 in the "BD+RISA" protocol.

For BD we were unable to determine the location of the irrecoverable fraction. This may be explained by technical limitations, since we could not detect BD in blood even when we added it to a sample of whole blood in vitro at a concentration of 2 $mg.ml^{-1}$; nor was BD detectable when the blood sample was centrifuged and the serum was analysed for BD. In the case of RISA, very low I^{125} gamma activity was found in decreasing order of activity per gram of wet tissue weight in fetal thyroid, fetal lung, fetal liver, fetal blood and maternal thyroid.

4.4.2 Interaction between tracers

Effect of RISA on BD binding

To test whether the binding of BD (38.5 ± 4.0%) might be dependent upon the presence of RISA, we performed a single experiment at G142 using only BD as the tracer ("BD-only"). In this experiment, 48.1% of the BD introduced at the start of the experiment was released by 5% BSA wash-out, a level of binding within the range observed in the "BD+RISA" protocol.

Effect of BD on RISA binding

To test whether binding of RISA required the presence of BD, we compared the bound fraction of RISA at G142 obtained in the presence of BD ("BD+RISA" protocol) with the

same fraction determined in the absence of BD ("RISA-only" protocol). As shown in Figure 4-2, the proportion of RISA bound was approximately 8-fold less in the absence of BD compared with the proportion bound in its presence (1.3 ± 0.2% versus 9.8 ± 0.7%; $p < 0.05$).

4.4.3 Changing background absorbance

To assess whether the background absorbance of lung liquid is constant throughout the experiment, we measured absorbance in every third sample taken in Periods 1 and 2, and in every sample during Periods 3 and 4, in the "RISA-only" protocol at G142 (n = 7). Absorbance of lung liquid increased from a mean of 0.31 ± 0.05 absorbance units (A) in the sample taken at the experiment onset to a maximum of 0.63 ± 0.11 A by the third sample, by which time approximately 45 min of mixing had occurred. Thereafter a slow downward trend was noted (Figure 4-3). Interestingly, the observed increase in absorbance was present not only at 620 nm, but over the whole spectrum from 325-900 nm. Centrifugation of the samples for 60 min at 2000 x g reduced absorbance of all samples to values ranging from 0.18 to 0.23 A at 620 nm (Figure 4-3); this range is close to the absorbance of water (0.18 ± 0.01 A; mean ± SD), saline (0.18 ± 0.01 A) and 5% BSA (0.19 ± 0.01 A). Centrifugation for 30 min or less at 2000 x g, however, did not completely remove the increase in background that occurred during the course of an experiment, while centrifugation for 10 min at 250 x g, as used in some studies employing BD for lung liquid volume determination (Garrad-Nelson and Perks, 1990; Kindler *et al*, 1993), reduced the rise in background absorbance by only about 50%.

To determine whether BD binds to the material causing a rise in background absorbance, we added BD to lung liquid samples with high background absorbance and measured

absorbance before and after centrifugation. This procedure was performed for sample L3 of each experiment in the "RISA-only" protocol. The reduction in absorbance obtained by centrifugation in the presence of BD (0.44 ± 0.12 A, n = 7) was not different from the reduction obtained by centrifugation before adding BD (0.44 ± 0.11 A, n = 7), indicating that BD did not spin down with whatever material is responsible for the rise in background absorbance.

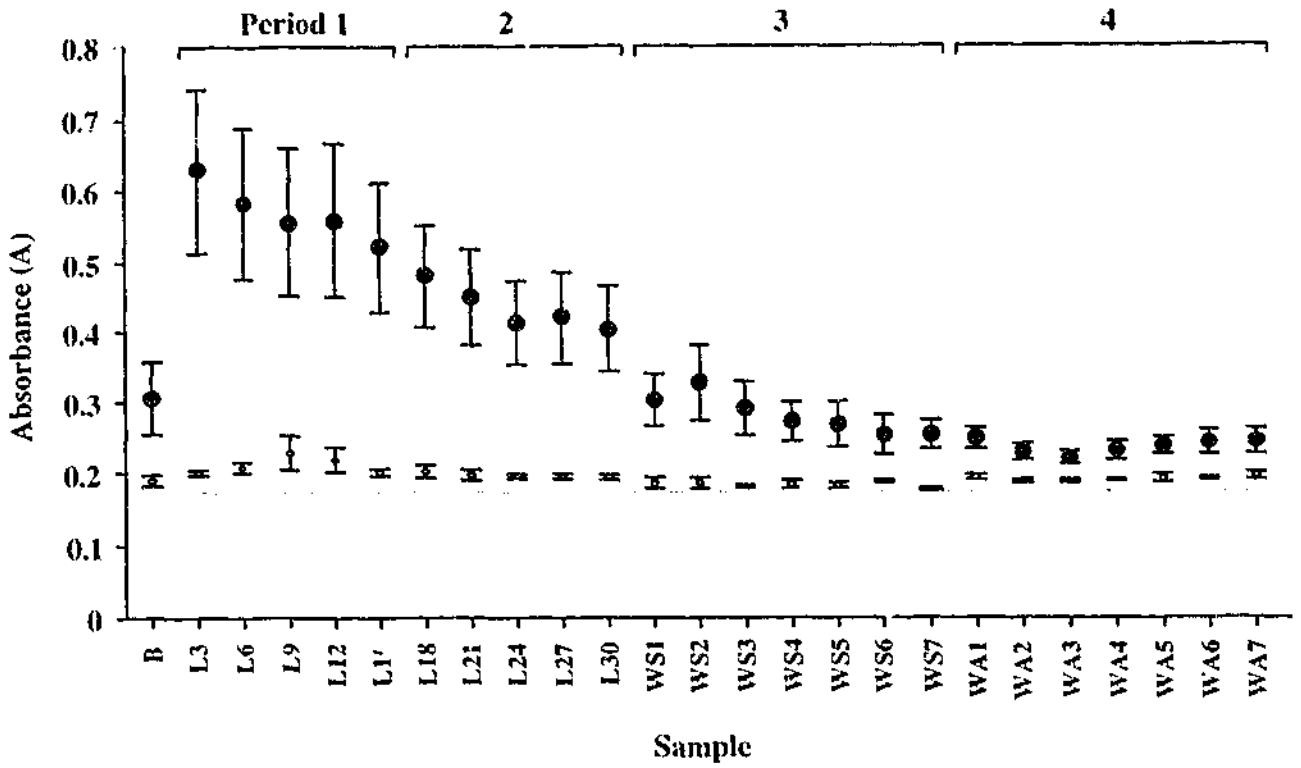

Figure 4-3. *Background absorbance (mean ± SE) in lung liquid samples taken in the "RISA-only" Protocol. The X-axis displays sample number; B represents the sample taken at experiment onset for determining background absorbance; L3-L30 are the third to the thirtieth samples taken for lung liquid volume determination; WS1-WS7 are samples of the seven saline washes, and WA1-WA7 are samples of each 5% albumin wash. Note that background absorbance of lung liquid increased considerably (filled circles) after the sample for background determination was taken. This background was almost entirely removed by spinning for 60 min at 2000 x g (open circles), so that it approximated the absorbance of water (0.175 ± 0.007 A), as indicated by the line at the bottom of the figure.*

4.4.4 Estimates of lung liquid volume and secretion rate

In the "BD+RISA" protocol, at G124 (n = 5) lung liquid volumes calculated without correcting for the fraction of tracer that is bound, or for changes in background absorbance, were 29.3 ± 3.9 ml.kg^{-1} for BD and 29.3 ± 3.2 ml.kg^{-1} for RISA. By contrast, at G142 (n = 7) calculated values using BD were more than 60% greater ($p < 0.05$) than those using RISA (36.3 ± 8.7 compared with 22.3 ± 3.5 ml.kg^{-1}; Figure 4-4).

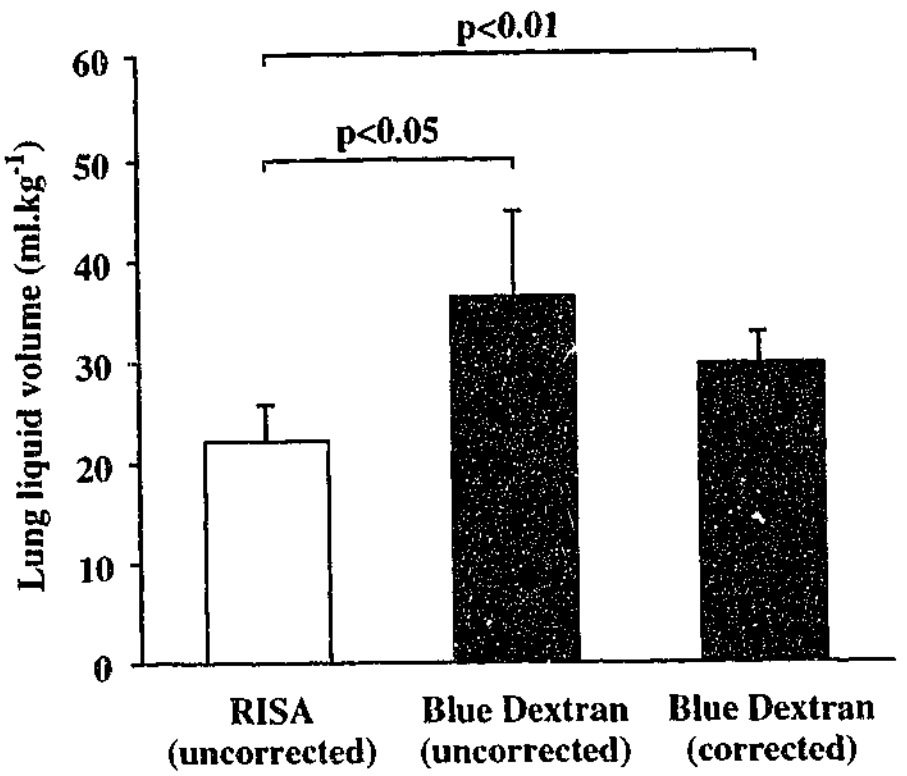

Figure 4-4. *Effect of BD binding to the epithelium, and the rise in background absorbance, on estimated lung liquid volume in ml.kgBW^{-1} determined at G142 in the "BD+RISA" protocol. Estimated volume derived with RISA is shown on the left, together with two estimates of volume derived with BD. The first BD value (uncorrected) was calculated without allowing for the fractions of BD that are bound or irrecoverable, or allowing for the rise in background. The second BD value was calculated using only the free BD and the absorbance after centrifugation.*

The volumes given above with each tracer were obtained with comparable methodology to those reported in all other studies using these tracers in the fetal sheep. However, it is also useful to examine how correction for bound and irrecoverable tracer affects estimated lung liquid volume. When BD concentrations were corrected for the background effect and for the fact that only a fraction of the added tracer is "free", volumes obtained with BD at G124 (31.5 ± 2.8 $ml.kg^{-1}$) did not differ significantly from volumes obtained with RISA. By contrast, at G142 calculated volumes using BD (29.7 ± 3.1 $ml.kg^{-1}$) remained greater than those using RISA by more than 30% ($p < 0.01$: see Figure 4-4).

4.4.5 BD + RISA in acute preparation

As in the BD+RISA protocol in chronically-prepared fetuses, a substantial proportion of each tracer was bound in 3 acute studies performed immediately after sterile operation (Table 4-2). This finding demonstrates that lung infection is not responsible for BD binding in our chronically-prepared fetuses. Lung liquid volume determined using the wet and dry weights of the lung, and from indicator dilution using BD and RISA, are also shown in Table 4-2. When lung liquid volume was calculated without correcting for the bound and irrecoverable tracer, both tracers grossly overestimated lung liquid volume as determined from wet and dry lung weights. When only the "free" amount of tracer was used in calculating the volume of liquid in the lung just before killing the fetus, in each of the 3 fetuses studied the volume derived with BD was greater than with RISA, but there was no statistical difference between the two estimates (Table 4-2). Although further data would be needed before reaching a conclusion, in this limited series the volumes estimated with both tracers were not significantly different from those calculated from lung weight (Table 4-2).

Table 4-2. Bound fractions of BD and RISA in 3 acute experiments, together with lung liquid volume estimated from lung weight and from uncorrected and corrected BD and RISA values

			ESTIMATED LUNG LIQUID VOLUME (ml)				
Fetus #	**BD bound**	**RISA bound**	**From lung weight**	**BD uncorrected**	**RISA uncorrected**	**BD corrected**	**RISA corrected**
9912	0.37	0.25	68.5	226.2	131.5	93.8	88.0
101	0.39	0.12	129.3	296.3	187.0	191.7	142.1
102	0.30	0.15	79.2	148.0	138.9	110.5	106.2
Mean	0.35	0.17	92.3	223.5	152.4	132.0	112.1
SEM	0.03	0.04	18.7	42.9	17.4	30.2	15.9

4.5 Discussion

Two lines of evidence support the idea that prenatal clearance of liquid from the fetal lungs is important for postnatal gas exchange. First, newborn infants born by elective caesarean section frequently have an excess of liquid in the lungs and typically develop a form of respiratory distress that is usually referred to as "wet lung" (Robert *et al*, 1976; Hales *et al*, 1993; Morrison *et al*, 1995). Second, physiological experiments show that removal of approximately half the liquid present in the fetal lungs before caesarean delivery speeds the normal rise in arterial O_2 levels that occurs immediately after birth (Berger *et al*, 1998).

Although it is generally accepted liquid is cleared from the lungs before delivery, just when this clearance occurs is under debate. In one study it was reported that lung liquid volume begins to decline in the last days of gestation in the lamb (Dickson *et al*, 1986), suggesting that the process of adapting the lungs for postnatal function is triggered before

labour. By contrast, recent reports present evidence that the volume of liquid in the fetal lungs continues to rise until the day before labour (Hooper and Harding, 1995; Harding and Hooper, 1996; Lines *et al*, 1997). On the basis of these reports, the mechanism that results in a decline in lung liquid before delivery would be initiated only after the start of labour. Such starkly differing results led us to test whether the discrepancy might be explained by the only methodological difference between the studies, with one using RISA as the volume tracer (Dickson *et al*, 1986), the others using BD (Hooper and Harding, 1995; Harding and Hooper, 1996; Lines *et al*, 1997). We hypothesized that either RISA underestimates volume in late gestation, or that BD overestimates it. Our results clearly show that when BD and RISA are used together at G124 the two tracers produce similar estimates of lung liquid volume. By contrast, in chronically prepared fetuses at G142 lung liquid volume estimated with BD exceeds that derived from RISA by an average of 63%.

We show that this overestimate springs from a number of weaknesses in the BD technique, the chief one being that a large proportion (average of 38.5%) of the BD added at the start of the experiment binds to the interior of the lung. When used in the presence of BD, a much smaller proportion of RISA (9.8%) also binds to the pulmonary epithelium, an effect that would cause overestimation of lung liquid volume derived from RISA. However, as we argue later, this seems likely to result from the radio-iodinated albumin binding to the epithelium in association with BD, and when RISA is used alone only a tiny amount (1.3%) of radio-label binds to the epithelium. Finally, the results obtained in 3 acutely prepared fetuses demonstrate that the high binding of BD we report in near-term fetuses is not associated with infection.

Our findings show that in our fetuses at G142, BD fails to satisfy a key requirement of the

indicator dilution technique; that is, the tracer must remain freely available in the compartment being studied, so that its dilution accurately reflects the volume of liquid in that compartment. We have also shown that the use of BD near term does not satisfy a second major requirement of the indicator-dilution technique, namely that the background level of the chosen tracer must remain constant over the course of an experiment. This background effect is most easily seen from the results obtained when samples taken in the "RISA-only" protocol were analysed for absorbance at 620 nm. Such changes in background absorbance after the start of an experiment may have a substantial effect on the estimated lung liquid volume. The magnitude and direction of the effect depend on the extent of mixing before the first sample is taken for measurement of background, since it is this value that is subtracted from all later samples. In addition, whether the sample is centrifuged will also determine the size of the error in the volume estimate. Since reports generally provide no information on either issue, the size of the problem in earlier publications is impossible to assess.

The two main sources of error inherent in the use of BD as a volume tracer in the lung of the late gestation lamb fetus are very large. These errors, however, have potentially opposing effects upon estimated lung liquid volume, with binding giving rise to an overestimate of lung liquid volume, and increased background giving rise to a potential underestimate of volume. Accordingly, by chance some estimates of lung liquid volume reported in studies using BD in late gestation might be close to the correct value. We may obtain insight into the magnitude of the error arising from binding and changing background by examining the difference between volume estimates derived from RISA and those derived from BD, either with or without correction for the two errors. As shown in Figure 4-4, when no correction is applied, estimated lung liquid volume derived with

BD at G142 exceeds the volume derived with RISA by an average of 63%. It is therefore clear that under our experimental conditions the binding effect exceeds the tendency for volume to be underestimated as a consequence of the background effect. Allowing for both sources of error, that is by using the absorbance of centrifuged samples, and by using only the free fraction of BD in calculations, estimated lung liquid volume at the time the trachea was first connected to the burette at the start of the experiment still exceeds that derived from RISA by more than 30%.

While the foregoing analysis provides insight into the magnitude of the errors that result from use of the BD technique near term in the fetal sheep, a number of issues remain. It is not obvious why the free fraction of BD and RISA can differ considerably at G124, and yet the volumes estimated with the two tracers do not differ significantly. Equally, it is not obvious why volumes calculated with BD at G142, after correcting for the two errors, do not equal those derived with RISA. One factor that may be important is the kinetics of BD binding to the epithelium. For example, slow binding throughout an experiment would mimic a high secretion rate, steepening the slope of the line that is subtended back to the start of the experiment to estimate lung liquid volume, thereby lowering the estimated lung liquid volume. This possibility is supported by our finding in acute preparations that BD, after allowing for bound and irrecoverable fractions, gives rise to lung liquid volume estimates reasonably similar to those derived from RISA at the end of the experiment i.e. after binding is likely to be complete (Table 4-2).

An explanation must also be given for the observation at G142 that 9.8% of the RISA added to the lung is released by wash-out with 5% BSA when RISA and BD are used simultaneously, and yet, when RISA is used alone, only 1.3% of the added RISA is

released by albumin wash-out. We propose that this finding is a consequence of the well-known high affinity of BD for proteins (Travis and Pannell, 1973; Angal and Dean, 1977; Dean and Watson, 1979; Gianazza and Arnaud, 1982) resulting in a molecule of BD binding reversibly both to the pulmonary epithelium and to the albumin of the RISA tracer. In this scenario, BD would remove RISA from lung liquid during the period of volume determination, followed by the release of both molecules by competitive displacement when unlabelled albumin in higher concentration (5%) is used to wash out the lungs. In the "RISA-only" protocol, the sites of attachment to the lung provided via BD are not available, and any binding of RISA to the epithelium must be direct; under these conditions the bulk of any RISA that binds to the epithelium will derive from the "cold" albumin added along with the RISA tracer at the start of the experiment.

A final issue for explanation is that the only previous study that fully documents a comparison of BD and RISA as volume tracers in the fetal lung reported that they produced similar volume estimates (Beierle *et al*, 1996). There may be a number of explanations for this result, one being that many of the fetuses used were studied earlier than G142. Although our results allow us to say no more than that the increased binding occurs at some time between G124 and G142, it is conceivable that binding and background errors remain small until fetuses approach G142. In addition, in the earlier validation study (Beierle *et al*, 1996) many of the fetuses, and perhaps all of them, were studied at more than one gestational age. When multiple studies are carried out, most of the epithelial binding sites for BD may already be occupied when BD is introduced into the lung liquid at the start of an experiment, with the result that almost all of the added BD might remain free within the liquid compartment. Under such circumstances, BD could provide an acceptable estimate of volume if the background effect were also minimized by

the design of the experiment. For example, if the protocol called for lung liquid to be thoroughly mixed before the first sample was removed for determination of background absorbance, background absorbance might already have reached its peak value (see Figure 4-3) before addition of BD. Interestingly, even if multiple studies were performed on each fetus, the high affinity of BD for albumin would result in some of the RISA added during each experiment binding to the epithelium in association with BD, giving rise to an overestimate of volume with RISA.

The large increase in BD binding to the pulmonary epithelium between G124 and G142 demonstrates that binding sites increase in number or affinity between these ages. Given the high affinity of BD for proteins, higher binding could result from an ontogenetic increase in cell surface proteins (Miller *et al*, 1989; Joyce-Brady and Brody, 1990) and these may include epithelial Na^+ channels which are induced by cortisol and TRH near term in the fetal sheep (Barker *et al*, 1991).

While the biggest error in the BD technique results from the bound fraction, both BD and RISA introduce error to volume estimates through a fraction that is irrecoverable by wash-out. In the "BD+RISA" protocol this irrecoverable fraction for both tracers was less than 10%, giving rise to a theoretical volume overestimate of approximately 11%. Where the irrecoverable tracer is located proved impossible for us to determine for BD, since we could not demonstrate its presence in blood. Thus, the statement that there is no BD measurable in blood in other studies (Cassin and Perks, 1982; Perks and Cassin, 1985b; Hooper *et al*, 1988; Cassin *et al*, 1994) cannot be used as evidence that BD is confined entirely within the lung compartment. For RISA we established that radio-label was present in fetal blood and a number of fetal and maternal tissues, including the thyroid,

after RISA is added to the fetal lung liquid. Whether this label was in the form of free iodine or was still bound to albumin we did not attempt to determine, but it represented only 4.9 ± 0.8% of the added tracer in the "RISA-only" protocol, and it would therefore introduce only a small error to volume estimates.

In summary, we have shown that when used alone, RISA satisfies the assumptions of the indicator dilution technique, both at G124 and near term. By contrast, BD binds strongly to the pulmonary epithelium of the fetal sheep near term. This binding, together with the rise in background absorbance that occurs during experiments, make BD unreliable as a tracer for lung liquid volume in the near-term fetal sheep, at least under the experimental conditions we used. Thus, the important question of whether lung liquid volume declines before labour, or continues to rise until labour begins, now needs to be carefully re-examined.

CHAPTER FIVE

VOLUME AND SECRETION RATE OF LUNG LIQUID IN THE FINAL DAYS OF GESTATION AND LABOUR IN THE FETAL SHEEP

VOLUME AND SECRETION RATE OF LUNG LIQUID IN THE FINAL DAYS OF GESTATION AND LABOUR IN THE FETAL SHEEP

5.1 ABSTRACT

Most of the liquid that fills the lung of the fetal sheep in late gestation is cleared by the end of labour. Clearance of this liquid has a beneficial effect on postnatal gas exchange and therefore represents an important adaptation for postnatal life. Despite its importance, there is disagreement about whether clearance begins prior to labour, or occurs entirely within labour. To address this issue, we made serial determinations of lung liquid volume by indicator dilution during late gestation and labour in the fetal sheep. Regression analysis demonstrated that liquid clearance occurred in two phases, the first of which began 70 hours before the fetus was in advanced labour. The second phase occupied the last 8 hours of the study period which was terminated when the ewe was in labour with active pushing. In the initial phase, average lung liquid volume fell from 38.3 $ml.kg^{-1}$ to 26.4 $ml.kg^{-1}$ before a rapid decline in the second phase reduced volume to 13.8 $ml.kg^{-1}$. The rate of lung liquid secretion also declined in two phases, both of which commenced earlier than the changes in lung liquid volume.

We conclude that clearance of lung liquid begins well before commencement of labour in the term fetal sheep, and then accelerates once labour is established. In our study, lung liquid volume fell even in the absence of reabsorption of liquid across the pulmonary epithelium, indicating that other mechanisms predominate in liquid clearance before birth.

5.2 Introduction

The fetal lung secretes a liquid which distends the future airspaces and plays a crucial role in promoting lung growth. Although essential for normal fetal development, both experimental (Berger *et al*, 1996) and clinical (Hales *et al*, 1993; Morrison *et al*, 1995) evidence supports the view that prenatal clearance of lung liquid before birth is critical for the establishment of normal respiratory function immediately after delivery. It is now clear that the vast bulk of this liquid leaves the lung before birth (Berger *et al*, 1998) and that at least a portion of the clearance occurs during labour (Brown *et al*, 1983) when rising concentrations of circulating adrenaline trigger liquid absorption by stimulating an active reabsorptive process mediated by trans-epithelial Na^+ transport. However, evidence is conflicting as to whether there are other mechanisms that bring about a decline in lung liquid volume before the onset of labour, thereby contributing to liquid clearance before birth. An early study reported evidence that lung liquid volume starts to decline several days before labour (Dickson *et al*, 1986), whereas data presented in a number of recent reports suggest that lung liquid volume continues to rise until the day before labour, reaching an average value as high as 50 $ml.kg^{-1}$ of body weight (Hooper and Harding, 1995; Harding and Hooper, 1996; Lines *et al*, 1997).

Both sources of evidence are open to criticism. The study that reported a pre-labour decline in lung liquid volume did not monitor uterine muscle activity (Dickson *et al*, 1986), leading to the suggestion that the animals may have been in early labour at the time a decline in lung liquid volume was observed (Lines *et al*, 1997). In the later series of studies (Hooper and Harding, 1995; Harding and Hooper, 1996; Lines *et al*, 1997), Blue Dextran was used as the impermeant tracer for lung liquid volume determinations. It has now been shown that a substantial proportion of this agent binds to the pulmonary epithelium of the

near-term fetal sheep (Pfister *et al*, 1999). Accordingly, use of this tracer may lead to a substantial overestimate of lung liquid volume, potentially giving rise to an artefactual rise in lung liquid volume in late gestation.

The experiments reported here were undertaken to determine conclusively whether lung liquid volume declines over the last days of gestation before the onset of labour, or whether the decline is restricted to labour itself. As the impermeant tracer we used radio-labelled human serum albumin (RISA), because we have shown that it does not bind in any significant extent to the pulmonary epithelium (Pfister *et al*, 1999).

5.3 Materials and Methods

5.3.1 Surgery

All surgical and experimental procedures conformed with the guidelines established by the National Health and Medical Research Council of Australia and had the approval of the Standing Committee in Ethics in Animal Experimentation of Monash University.

Operations were performed on twelve fetuses of pregnant Border-Leicester ewes between 131 and 133 days gestation (G131 to G133; term = G147). Anaesthesia was induced with an intravenous injection of propofol (5 mg kg^{-1}, Zeneca Ltd., Macclesfield, United Kingdom) and maintained with a mixture of 1-2% halothane, 66% N_2O and 32-33% O_2. The maternal abdomen was opened in the midline and the fetal head was delivered through a uterine incision. After a midline ventral opening was made in the fetal neck, the carotid artery was catheterized non-occlusively using a teflon cannula-tygon tubing assembly. Two wide-bore silastic catheters (ID 3 mm, OD 6 mm; Sil-Med Corporation, Taunton, Massachusetts) were introduced through a single tracheostomy, with one catheter directed

rostrally so that its tip lay 2 cm or more from the larynx, while the other catheter was directed caudally towards the lung. The two free catheter ends were connected to form a liquid-filled loop. The skin incision was sutured and a liquid-filled catheter open to the amniotic space was secured to the skin nearby. The thorax was opened at the level of the 9th intercostal space to expose the diaphragm and bipolar harpoon EMG electrodes (Cooke *et al*, 1990) were inserted into the diaphragm for recording electromyogram activity (EMG_d). The intercostal space was then closed with a silk ligature. Harpoon electrodes were also inserted into the external oblique muscle of the abdomen and into the external intercostal muscle of the 6th interspace after exposing each muscle through small skin incisions.

The fetus was returned to the uterus which was carefully closed to prevent leakage of amniotic fluid. Finally a bipolar harpoon EMG electrode was pushed into the uterine muscle (EMG_u) and its lead was sutured to the uterus. The ewe's abdominal wall was sutured and all catheters and wires were tunnelled subcutaneously to her flank where they exited through a small incision and were stored in a bag secured under a stocking encircling the abdomen. All animals received post-operative analgesia (50 mg Finadyne, Schering-Plough, Australia) and daily intramuscular antibiotic treatment (500 mg procaine penicillin, 500 mg dihydrostreptomycin). Animals were allowed to recover for 7 to 9 days before observations commenced.

5.3.2 Estimation of fetal body weight

All fetuses were studied longitudinally over a number of days. Fetal body weight was calculated for each gestational age studied, using published fetal sheep growth curves (Lumbers *et al*, 1985; Davis *et al*, 1992), corrected for each fetus with its post-mortem

weight according to the following formula:

$$FBW = FBW_{ave} * (FBW_{PM}/FBW_{avePM})$$

where FBW is the desired weight for a particular fetus and gestation, a value that is calculated from FBW_{ave}, the average weight of a sheep fetus at that gestation taken from sheep growth curves, multiplied by the ratio of FBW_{PM} (actual weight of the fetus at postmortem) divided by FBW_{avePM} (average fetal weight from sheep growth curves at the gestational age at termination of the experiment). The average weights used for each gestational age were derived from the mean of the two published formulae (Lumbers *et al*, 1985; Davis *et al*, 1992) which provided comparable values with a difference between two estimates of 2.8 ± 0.5% (mean ± SEM).

5.3.3 Experimental protocol

On the day of each experiment, fetal carotid and amniotic fluid catheters were connected to pressure :ransducers (Hewlett-Packard 1280) and amplifiers (Hewlett-Packard 8805B). The diaphragm, external oblique and external intercostal EMG electrodes were connected to differential amplifiers (Neomedix NT114) and band-pass filtered (40 Hz - 2 kHz). All signals were displayed continuously on a chart recorder (Neotrace 800Z, Neomedix), digitized using a Maclab/16S system (ADInstruments, Castle Hill, NSW, Australia) and stored on a personal computer.

5.3.4 Measurement of pressure in the future airspace of the lung

During postmortem examination of near-term fetuses in which a tracheal loop had been implanted, we have occasionally found a thick mucus plug near one or both cannula tips,

suggesting that the loop could become obstructed in some fetuses. We reasoned that an obstruction anywhere in the tracheal loop would dam liquid in the lung and raise hydrostatic pressure within the lung. At the start of each experimental day we therefore measured pressure in the lung end of the tracheal loop and corrected this value by subtracting amniotic fluid pressure (P_{AF}) measured at the same time.

5.3.5 Determination of lung liquid volume and secretion rate

Under sterile conditions, the lung end of the tracheal loop was connected to a temperature-controlled (40°C) glass burette that was sealed at its top with a rubber stopper. An 18-gauge needle was passed through the stopper and a Millipore filter was attached to the needle. With this arrangement, atmospheric pressure and sterility were maintained in the burette. We drained liquid from the lung into the burette and reinstilled it into the lung for a period of 30 min before taking a sample (0.5 ml) to measure its background radioactivity; this step was omitted for the first experiment in each fetus, since there was no radioactivity present in the lung at the start of this experiment. A volume of 50 - 100 ml was then collected in a container, weighed and a known quantity of RISA thoroughly mixed into it (3 - 8 μC for measurements separated by one day or more; 10 - 20 μC when a second measurement was made on the day of labour). In addition, 0.5 - 1 ml of fetal serum was added as a source of "cold' albumin, before a small sample was taken to determine its level of radioactivity. The remaining liquid was weighed accurately (Mettler AE 166 delta range), returned to the burette and re-instilled into the lung. The tracer was mixed with lung liquid by repeated cycles of drainage and re-instillation over at least 30 min, using a maximum hydrostatic pressure of ± 15 cm H_2O. After 30 min of mixing, 12 - 15 samples of 0.5 ml were taken at intervals of 5 - 10 min whilst cycling was continued. In one animal, due to concern that labour was progressing rapidly, only two samples were taken in

advanced labour, allowing us to estimate lung liquid volume (V_L), but not secretion rate (J_v).

Concentration of RISA in each sample was measured on a gamma counter (1282 Compugamma, LKB, Wallac; window set between 20 and 100 keV). Lung liquid volume and secretion rate were calculated according to established and verified techniques (Brown *et al*, 1983; Pfister *et al*, 1999). J_v, based on the slope of the relationship between lung liquid volume and time, was calculated using the least squares method. V_L at the time of interruption of the tracheal loop ($V_{Lt=0}$) was obtained by extrapolation.

During the course of each experiment, several samples of carotid arterial blood were taken to analyse fetal blood gas and acid-base status at an assumed body temperature of 40°C (Radiometer ABL500, Radiometer, Copenhagen) and to measure haemoglobin concentration and O2 saturation (OSM2 Hemoximeter, Radiometer, Copenhagen).

Lung liquid secretion rate and lung liquid volume were determined longitudinally in each animal, with the first experiment performed at G140 (in one fetus the first study was performed at G141) and every 1 - 2 days thereafter until the detection of labour. Continuous monitoring of uterine activity (EMG_u), P_{AF} and fetal breathing activity (EMG_d) was instituted at G142. This allowed recognition of the early signs of labour (see later) and an immediate determination of V_L and J_v was performed. Another determination was made 7 - 24 hours later unless the appearance of fetal parts and vigorous maternal pushing suggested there was insufficient time to complete the experiment. After the final labour determination was made, the ewe was euthanased with an overdose of anaesthetic (Lethabarb, Virbac, Peakhurst, NSW, Australia). A postmortem examination was

performed, checking for signs of fetal infection by inspecting the appearance and smell of the fetus, placenta and amniotic fluid, as well as its internal organs and fluids. The fetus was weighed and the lung was dissected before placing it in an oven at 95°C for approximately 2 weeks until it reached a constant dry weight.

5.3.6 Definition of labour

As recognition of labour was crucial to the study, animals were monitored continuously from G142. Ewes were observed at regular intervals for clinical signs of impending labour, chiefly a reddening of the vulva and the appearance of mucus at the introitus. Behavioural changes were also carefully monitored, including decreased feeding and increased restlessness, as evinced by repeated change of posture from sitting to standing, and a frequent scraping of the forefeet on the floor of the pen. As the stages of labour are usually based on clinical findings and ill-defined (Crawford, 1983; 1985) we based our definitions on EMG_u and P_{AF} recordings.

Typically, in late gestation, but before labour was clear-cut, we observed infrequent and irregular short bursts of EMG_u activity with little or no increase in P_{AF} (Figure 5-1, left panel) similar to the contractions manually palpated and described in the human by John Braxton Hicks (Hicks, 1871). As the ewe entered labour, we observed increasingly regular bursts of uterine activity separated by inactive periods some 12 - 24 hours before fetal parts became apparent at the introitus. Early labour (approximating Stage I in the human) was diagnosed when synchronous amniotic fluid pressure waves of 5 - 15 cm H_2O accompanied EMGu activity on a regular basis (Figure 5-1, middle panel). Advanced labour (approximating Stage II in the human) was diagnosed when the ewe started active and repeated pushing, giving rise to amniotic fluid pressure peaks that reached > 20 cm

H_2O (Figure 5-1, right panel).

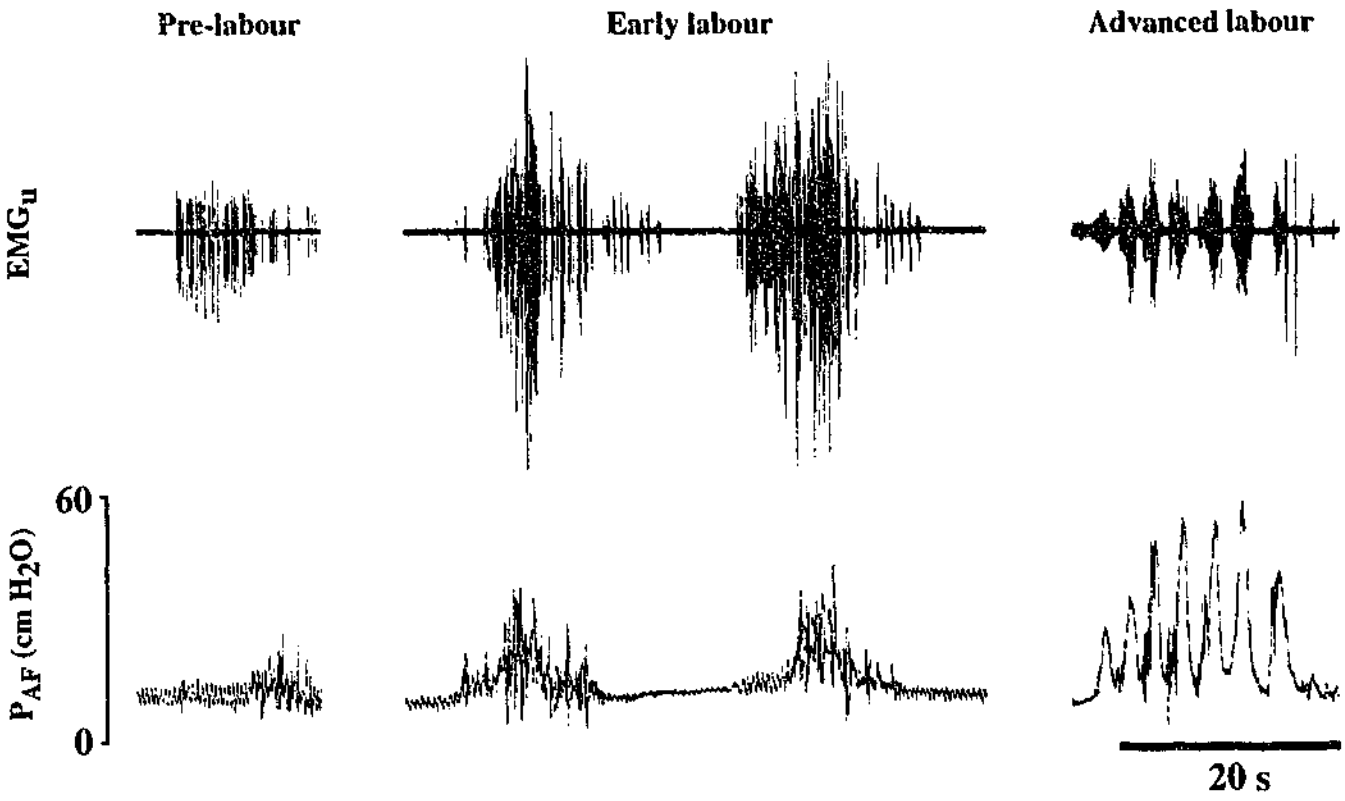

Figure 5-1. *Uterine activity and amniotic fluid pressure before and during labour. Uterine electromyogram (EMG_u) and amniotic fluid pressure (P_{AF}) from a fetus taken before labour (pre-labour), during early labour and in advanced labour. Before the establishment of labour, infrequent uterine contractions gave rise to a small increase in amniotic fluid pressure. In early labour, uterine contractions were more frequent and powerful, while in advanced labour maternal pushing was evident and it was sometimes in phase with the EMG_u as shown in this record.*

5.3.7 Exclusion criteria

The study was designed to be longitudinal, so it was decided at the outset that fetuses would be excluded unless lung liquid volume and secretion rates were determined at least once before and once during labour. In addition, since it was our intention to study only fetuses that had experienced a normal gestation and delivery, we decided to exclude fetuses if they entered advanced labour earlier than 141 days gestation (G141). This lower limit was chosen because lambs that deliver vaginally at this age or later manifest a normal rapid increase in arterial oxygen levels (Berger *et al*, 1990), suggesting all had experienced normal adaptations for birth.

5.3.8 Data analysis

The time at which a lung liquid volume and secretion rate determination was made during each study in a fetus was referenced to time zero, which was either the time the last determination began in advanced labour (8 of the 9 animals studied), or, in the fetus in which we did not perform an experiment in advanced labour, we took the time the ewe was killed. After establishing that the data were not skewed, we subjected the raw data to least squares regression analysis (SPSS, SPSS Inc., Chicago, Illinois). The variation attributable to animals was removed before examining the effect of time on lung liquid volume and secretion rate, the two dependent variables. We tested three models on the entire data set: first, a model in which each variable had a plateau level before a single slope decline occurred until time zero; second, a model in which a plateau level was followed by a two-slope decline in each variable; third, a model in which there was an increase in each variable before a decline to time zero. The first and second models were designed to test the claim that lung liquid volume declines before the onset of labour (Dickson *et al*, 1986), while the third model tested the claim that it continues to increase until the start of labour

(Hooper and Harding, 1995; Harding and Hooper, 1996; Lines *et al*, 1997). The analysis was also performed on the data after the early and advanced labour values were removed; however, in this analysis, we tested only the first and third models. In each of the analyses, the pivot points between the two or three segments of the relationship between each variable and time were shifted progressively along the time axis, and the positions giving rise to the minimum residual variation were accepted as providing the best fit to the data.

Volumes, secretion rates and lung weights were expressed per kilogram of body weight. Blood gas, haemoglobin and pH values were compared by one-way ANOVA after binning the data. When a fetus had more than one value in a bin we used the average value. For a number of comparisons we used the unpaired or paired Student's t-test as appropriate.

Values are given as mean ± SEM. Differences were considered significant when $P < 0.05$.

5.4 Results

Of the 12 chronically instrumented fetal sheep that entered the study, two went into early labour and one had an obstructed tracheal loop at G142, as confirmed at postmortem by the presence of a kink in the tracheal tubing close to the fetal neck. Blood gas, acid-base and haemoglobin levels over the final days of gestation and labour indicate that fetuses were healthy throughout the study period, and no significant change was found in any of the variables over the period of study (Table 5-1). Fetuses reached advanced labour at a mean gestational age of 145.4 ± 0.8 d when the study was terminated and fetal weight was taken (5.1 ± 0.2 kg). Postmortem examination revealed no abnormality in any of the fetuses. Dry lung weight (3.3 ± 0.1 $g.kg^{-1}$) was similar to that reported in an earlier study (Berger *et al*, 1998).

Table 5-1. Respiratory variables during the course of the study, showing data binned according to the timing of each study relative to advanced labour

	Pre-labour > 6 days (n = 3)	Pre-labour 3 - 6 days (n = 7)	Pre-labour 1 - 3 days (n = 8)	Early labour (n = 8)	Advanced labour (n = 8)
Hb (g dl^{-1})	11.3 ± 0.3	11.3 ± 0.3	11.9 ± 0.4	12.1 ± 0.6	11.7 ± 0.4
pH_a	7.35 ± 0.00	7.34 ± 0.01	7.35 ± 0.00	7.34 ± 0.01	7.33 ± 0.01
S_{a,O_2} (%)	53.5 ± 4.3	55.8 ± 2.1	52.6 ± 2.6	47.2 ± 2.3	43.7 ± 4.9
P_{a,O_2} (mmHg)	23.9 ± 2.6	24.8 ± 1.3	23.9 ± 1.4	22.8 ± 1.1	21.7 ± 1.3
P_{a,CO_2} (mmHg)	53.9 ± 0.8	53.9 ± 0.8	53.2 ± 0.8	55.1 ± 1.0	54.6 ± 1.3

Values are means ± SEM. Note that n signifies the number of fetuses contributing data to each bin.

Early and advanced labour were recognised, according to the definitions outlined in the Methods section, in all 9 animals. However, due to rapid progress, complete experiments in advanced labour were possible in only 7 of the 9 fetuses studied. In one of the remaining two fetuses, only two lung liquid samples were obtained before the experiment was terminated because of concern that delivery might occur; in this animal we could therefore estimate lung liquid volume (V_L) in advanced labour, but not secretion rate (J_v). In the second fetus, no determination of lung liquid volume or J_v was possible during advanced labour. The determination made in early labour preceded that in advanced labour by 15.4 ± 2.7 h.

5.4.1 Lung liquid volume

Between 3 and 8 determinations of V_L were made in the 9 fetuses studied. These values

spanned a period of approximately 8 days before termination of the experiment in advanced labour. The raw data for each fetus are shown in Figure 5-2, with values pertaining to each fetus connected by dashed lines. Variation attributable to animals was removed before fitting one of the three models tested. The first model incorporated a plateau level for V_L followed by a single slope regression.

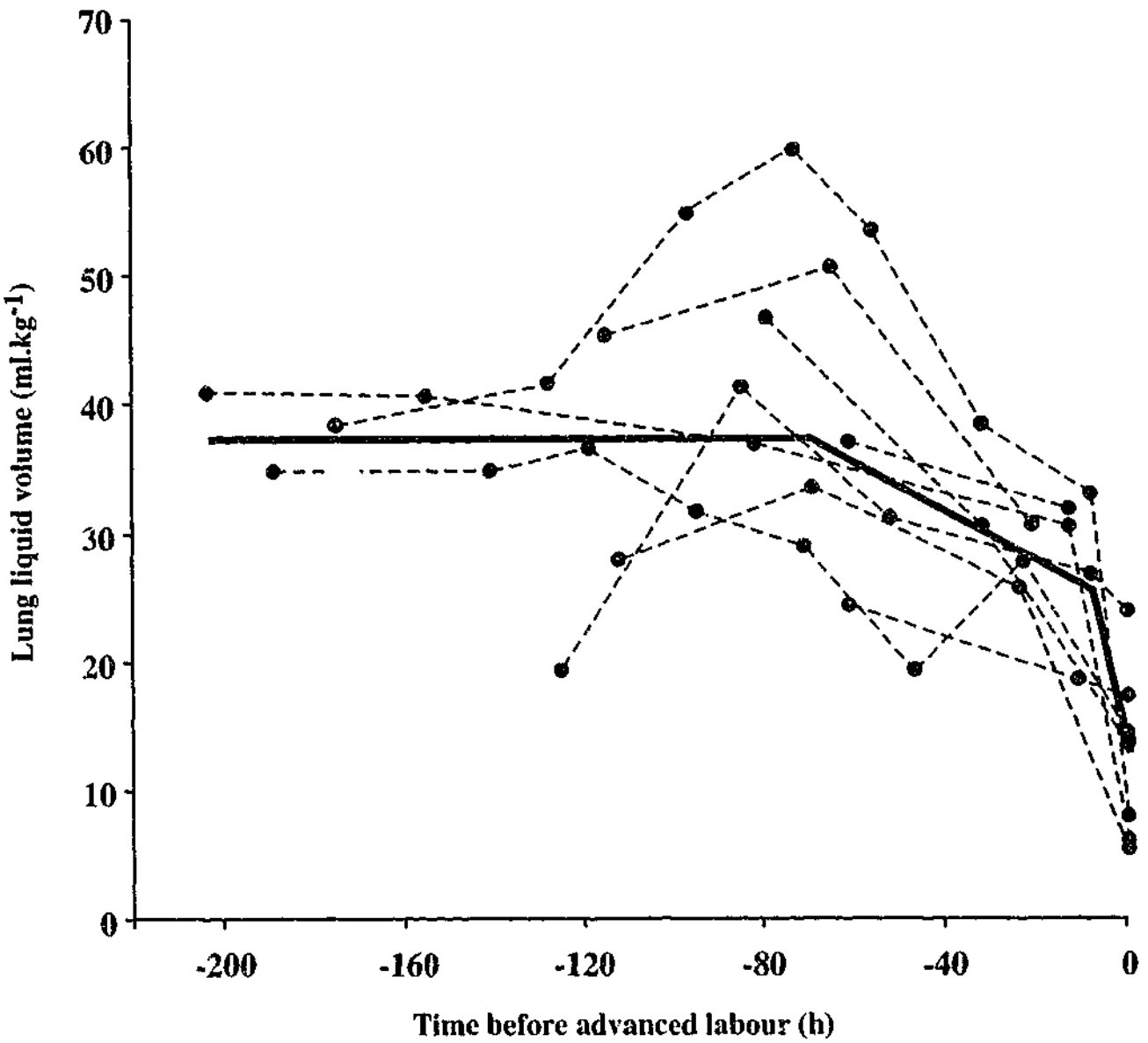

Figure 5-2. *Change in lung liquid volume in the last days of gestation and labour. Note that data points for each animal are joined by dashed lines, while the solid line shows the average lung liquid volume for all the fetuses, as derived from the regression analysis.*

Table 5-2. Analysis of variance for lung liquid volume (V_L) data in Figure 5-2

Source of variation in V_L	Degrees of freedom	Sum of squares	Mean square	*F*
Animals	8	1981	248	4.5
Time				
plateau + one-slope regression	2	2934	1467	26.7
incorporation of second slope	1	306	306	5.6
Residual	30	1651	55	
Total	41	6872		

The plateau + one-slope regression is significant at the 0.1% level. A significantly improved fit to the data was achieved by the incorporation of a second slope to the model ($P < 0.025$).

As can be seen from Table 5-2, this model accounted for a considerable proportion of the variation in the data, and the pivot point (the position on the time axis at which V_L began to decline) providing the best fit to the data was at 37 hours before time zero. A significant improvement in the data fit was achieved when a second slope was added to the model, as shown in Figure 5-2. The two pivot points giving the best fit to the data were at 70 hours and 8 hours before time zero. Based upon the regression, the average initial V_L was 38.3 $ml.kg^{-1}$ before it declined to 26.4 $ml.kg^{-1}$ in early labour and to 13.8 $ml.kg^{-1}$ in advanced labour. The initial rate of decline of V_L at 70 hours was 0.19 $ml.kg^{-1}.h^{-1}$, increasing to 1.58 $ml.kg^{-1}.h^{-1}$ over the last 8 h of labour studied. When we tested a model in which V_L increased initially before declining to time zero, analysis showed that the slope of the initial rise in V_L was not significantly different from zero and this model did not account for more of the variation in the data than the plateau plus 2-slope model. When only the pre-labour data were included in the analysis, we found that the best fit was achieved with

a plateau and a 1-slope model, with the pivot point at 56 hours before time zero.

5.4.2 Lung liquid secretion rate

Secretion rates are plotted in Figure 5-3 and again the values for individual fetuses are connected by dashed lines. After removal of animal variation, the J_v data were tested with the same three models used to fit the V_L data.

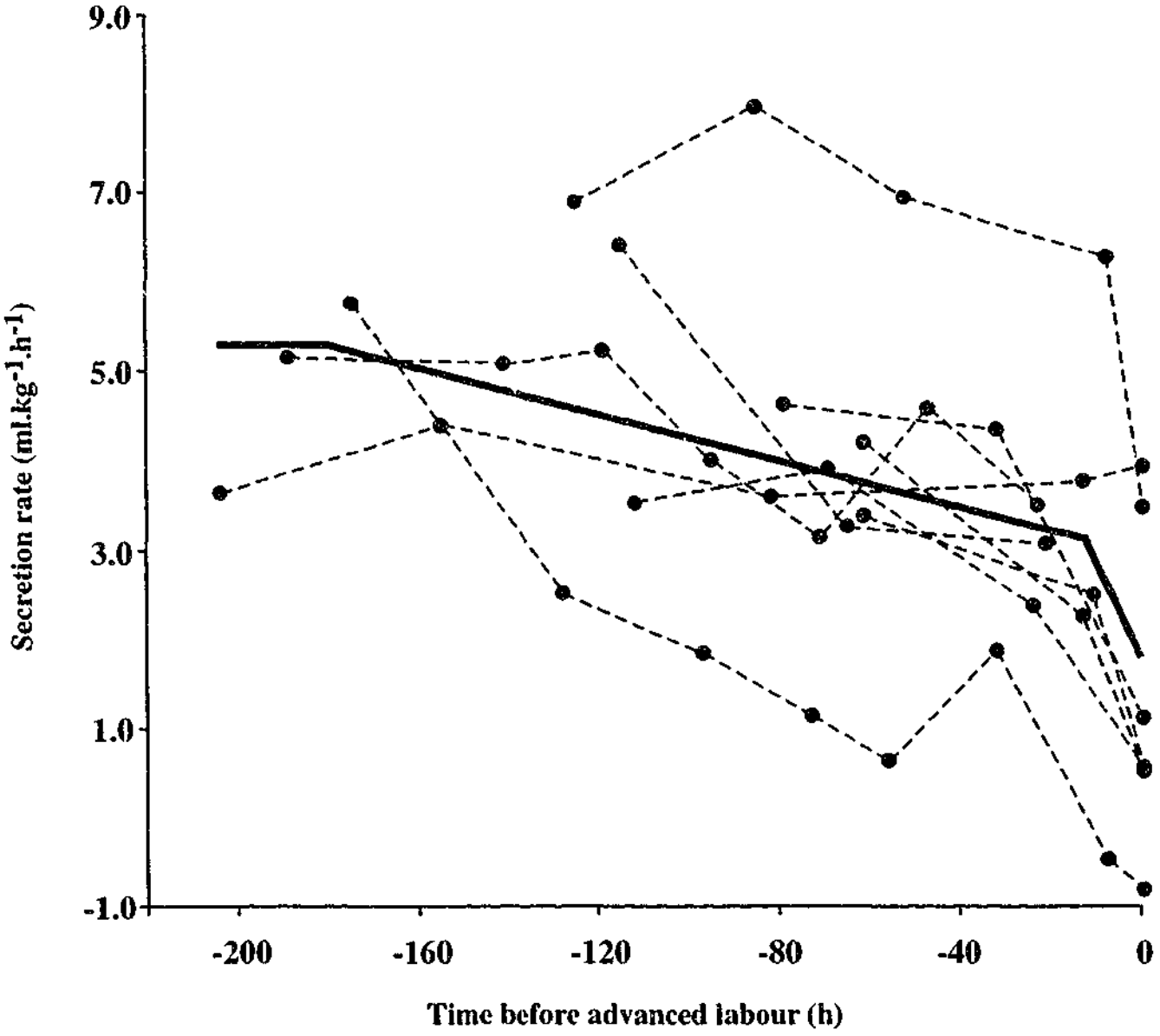

Figure 5-3. *Change in lung liquid secretion in the last days of gestation and labour. Values for each animal are joined by dashed lines. The solid line shows the average for the study group derived from regression analysis.*

Table 5-3. Analysis of variance for lung liquid secretion (J_v) data in Figure 5-3

Source of variation in J_v	Degrees of freedom	Sum of squares	Mean square	*F*
Animals	8	90.0	11.25	10.9
Time				
plateau + one-slope regression	2	40.9	20.45	19.9
incorporation of second slope	1	6.8	6.75	6.6
Residual	29	29.9	1.03	
Total	40	167.6		

The plateau + one-slope regression is significant at the 0.1% level. A significantly improved fit to the data was achieved by the incorporation of a second slope to the model ($P < 0.025$).

As seen in Table 5-3, the plateau plus one-slope model accounted for a considerable proportion of the variation in the data; the pivot point between the plateau and the single slope at which the residual error was minimised was at 33 hours before time zero. Addition of a second slope to the model gave a further significant improvement in fit, and the pivot points leaving the minimum residual error were at 180 and 12 hours before time zero (see Figure 5-3). In the first phase of its decline, average secretion rate fell from 5.3 $ml.kg^{-1}.h^{-1}$ to 3.1 $ml.kg^{-1}.h^{-1}$, before decreasing in the second phase to 1.8 $ml.kg^{-1}.h^{-1}$. As was the case for V_L, there was no evidence of an increase in J_v before it began to decline.

When the secretion rates in early and advanced labour data were removed from the data set, the best fit to the data was produced with a plateau and a one-slope decline to time zero. The pivot point in this reduced data set occurred at 180 hours before time zero. It was noteworthy that J_v measured in early labour (2.9 ± 0.8 $ml.kg^{-1}.h^{-1}$, $n = 8$) was significantly

greater than zero ($P < 0.05$) and that Jv in advanced labour (1.4 ± 0.7 ml.kg^{-1}.h^{-1}, $n = 7$) was not significantly different from zero; these values were not significantly different from one another. Only a single animal manifested reabsorption of lung liquid in our study group, and this animal reabsorbed liquid both in early (-0.4 ml.kg^{-1}.h^{-1}) and advanced labour (- 0.8 ml.kg^{-1}.h^{-1}).

5.4.3 Intra-pulmonary pressure and lung liquid volume

The relationship between lung liquid volume and intra-pulmonary pressure, measured immediately on opening the tracheal loop at the start of each experiment, is shown in Figure 5-4. Using all values, the calculated regression line ($y = 2.1 x + 32.2$; $r = 0.76$) predicted a lung volume of 32.2 ml.kg^{-1} at zero pressure. We also performed a regression analysis using only those data points with a zero or positive intra-pulmonary pressure; that is, we excluded negative values since these appear to fall on the lower limb of what looks like the normal sigmoid pressure-volume curve of the lung, except that the upper plateau is absent (Figure 5-4).

In this case, shown by the line in Figure 5-4, the calculated regression ($y = 4.1x + 28.8$; $r = 0.64$, $P < 0.001$) predicted a lung liquid volume of 28.8 ml.kg^{-1} at zero intra-pulmonary pressure, rising by 4.1 ml.kg^{-1}.cmH_2O^{-1}. In all advanced labour studies where we determined intra-pulmonary pressure ($n = 6$), we measured values of zero and below. In 3 of the 7 measurements made in early labour, the intra-pulmonary pressure was negative. One negative intra-pulmonary pressure value was obtained in a fetus 61 h before time zero. When the values during labour were excluded, the average intra-pulmonary pressure in the fetuses studied was 2.2 ± 0.5 cm H_2O.

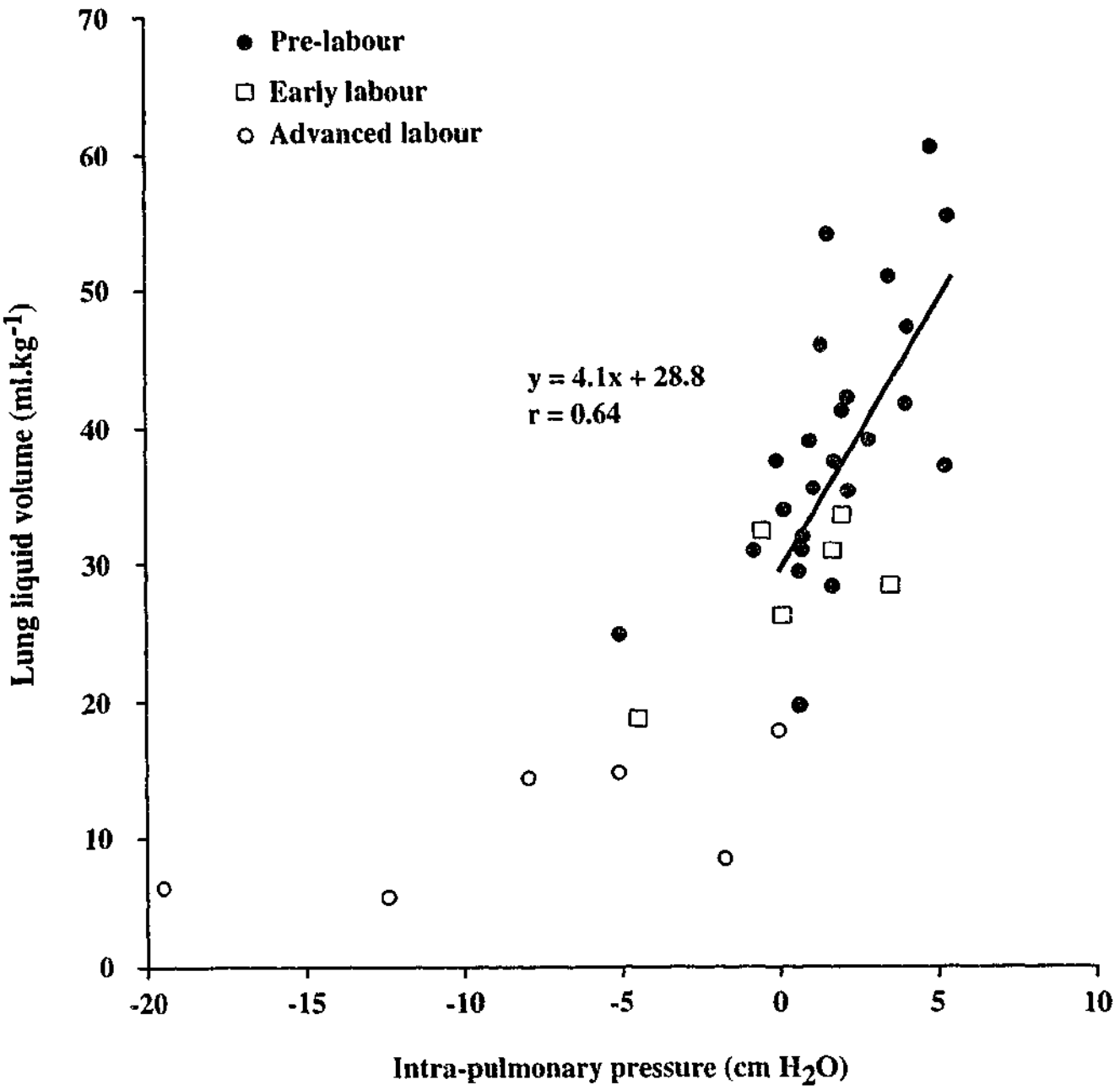

***Figure 5-4**. Relationship between lung liquid volume and intra-pulmonary pressure. The regression line shown was calculated using lung liquid volumes associated with intra-pulmonary pressures of zero and above.*

5.5 Discussion

Our key finding is that lung liquid volume exhibits a plateau level in late gestation before declining in two phases over a period of about 3 days leading up to and including the final stage of labour. In its initial phase, beginning considerably before the advent of labour, lung liquid volume declines gradually at a rate of 0.19 $ml.kg^{-1}.h^{-1}$. In a second phase that is restricted to labour, the decline in volume is much faster, averaging 1.58 $ml.kg^{-1}.h^{-1}$. The rate of lung liquid secretion showed a similar pattern of decline, although the timing of commencement (180 h), and the point of acceleration (12 h), were earlier than the changes in lung liquid volume.

Reabsorption of lung liquid occurred in only one of our fetuses whereas an earlier study showed that rapid reabsorption of liquid occurs during the last 1 - 2 h before delivery (Brown *et al*, 1983). In the current series of experiments, however, it is likely that observations were terminated before circulating adrenaline levels had risen sufficiently to initiate Na+ transport across the pulmonary epithelium (Olver *et al*, 1986). Nevertheless, the important insight generated by our results is that even before active reabsorption commences a very large decline in lung liquid volume has taken place. On average, we found that lung liquid volume falls by approximately 25 $ml.kg^{-1}$ between G140 and advanced labour, a volume that represents the major part of the 75% decline in lung liquid volume shown to occur before birth in previous work (Berger *et al*, 1998).

This and earlier studies (Brown *et al*, 1983; Berger *et al*, 1998) enable us to define the time course of changes in lung liquid volume in the days immediately before birth. Initially, volume declines relatively slowly, but persisting for a period of more than 2 days before birth, resulting in the removal of a large volume of liquid. During this initial phase, lung

liquid volume falls by approximately 30%, from 38.3 ml.kg^{-1} several days before birth to 26.4 ml.kg^{-1} in early labour. A more rapid fall then takes lung liquid volume to 13.8 ml.kg^{-1} by the time we terminated the study in advanced labour. Although we terminated the study at this point, it is highly likely that liquid clearance would have continued had we not done so. Indeed, Berger *et al*. (1998) showed that by the time of birth, the average volume of liquid in the fetal lung was only 6.8 ml.kg^{-1}, just half that at termination of the current study. We suggest that the final step in the clearance of lung liquid involves active reabsorption, a process that has been shown to be stimulated by the catecholamine surge of labour (Brown *et al*, 1983). Reabsorption of liquid from the lung is driven by active Na^+ transport wh' h then continues to play a dominant role in keeping the airspace dry throughout postnatal life (Ramsden *et al*, 1992).

5.5.1 Comparison with earlier work

Our results confirm an earlier demonstration that the volume of liquid in the fetal lung starts to decline before the onset of labour (Dickson *et al*, 1986). This report has been criticised because labour was not monitored with a uterine electromyogram, leading to the suggestion that fetuses may already have been in labour when the decline in lung liquid volume was first observed (Lines *et al*, 1997). No such criticism can be made of the current study, since uterine EMG and amniotic pressure were monitored continuously from G142. Even acknowledging the difficulty of precisely identifying the onset of labour, it is unlikely that the fetus was in labour when we first observed a decline in lung liquid volume, since this would require labour to have begun 70 hours before we terminated the study in advanced labour.

Our results run counter to the conclusion, based on a compilation of results from several

sets of experiments (Hooper *et al*, 1988; Hooper and Harding, 1989; Hooper and Harding, 1990; Wallace *et al*, 1990; Miller *et al*, 1993; Hooper and Harding, 1995; Wallace *et al*, 1995; Harding and Hooper, 1996; Wallace *et al*, 1996a; Wallace *et al*, 1996b; Lines *et al*, 1997), that lung liquid volume continues to rise until the day labour begins. By this time, the volume of liquid in the lung reportedly reaches an average level as high as 50 $ml.kg^{-1}$ (Harding and Hooper, 1996).

We suggest that two methodological problems could account for the disparate results. The first problem arises from the use of Blue Dextran as the impermeant tracer whose dilution is used to calculate volume in studies reporting a continuous increase in lung liquid volume until labour. This molecule has a high affinity for proteins (Dean and Watson, 1979) and binds to the pulmonary epithelium in substantial quantities (Pfister *et al*, 1999). Such binding, by reducing the quantity of free Blue Dextran in lung liquid, is likely to result in an overestimation of lung liquid volume. We have also shown that the magnitude of this overestimation may increase with gestation, as considerably more Blue Dextran binds at G140 than at G125 (Pfister *et al*, 1999).

A second potential source of error is one that is common to virtually all studies of lung liquid volume in the chronically catheterised fetal sheep. An exteriorised loop of tubing is implanted in the trachea to allow lung liquid to be sampled and its volume determined (Adamson *et al*, 1975). This tubing must be long and of narrow bore, its diameter being set by that of the trachea and a thick wall being needed to avoid kinking. Unavoidably, therefore, the tubing increases resistance to the outflow of liquid through the trachea, and may increase the distending pressure and volume of liquid in the lung. The presence of highly viscous mucous in the loop would accentuate this effect, and mucous masses are

commonly seen flowing in the tubing when liquid is drained from the lung at the start of experiments. The potential for the tracheal loop to increase lung liquid volume substantially is evident from Figure 5-4, which shows that in the positive pressure range the compliance of the lung is 4.1 $ml.kg^{-1}.cmH_2O^{-1}$, a value close to that reported earlier (Dickson and Harding, 1991). Consistent with our view that the tracheal loop leads to over- . flation of the lung, the average lung liquid volume in the present study at G140 (35.1 ± 3.1 $ml.kg^{-1}$) shows a trend to higher values ($P = 0.07$) than reported in 10 fetuses of the same age (28.2 ± 1.8 $ml.kg^{-1}$) in an earlier study which did not employ a tracheal loop (Berger *et al*, 1998).

5.5.2 Lung liquid secretion and reabsorption

Lung liquid secretion rate began to decline well in advance of labour, thereby confirming the conclusions of two earlier studies (Kitterman *et al*, 1979; Dickson *et al*, 1986). Relatively low rates of liquid secretion were reported in another study in the late gestation sheep and, interestingly, addition of amiloride to the lung lumen resulted in a significant increase in liquid secretion (Olver *et al*, 1986). Thus secretion may decline near term as a result of a progressive increase in active Na^+ transport competing with the Cl^- secretory mechanism.

5.5.3 Negative intra-pulmonary pressure in labour

For the first time we demonstrate a large negative intra-pulmonary pressure in advanced labour, indicative of lung volume being less than functional residual capacity. This finding may in part explain reports that the first inspiration in vaginally-delivered human infants does not require diaphragmatic contraction (Geubelle *et al*, 1959; Karlberg, 1960; Karlberg *et al*, 1962a; Karlberg *et al*, 1962b). Karlberg proposed that "vaginal squeeze", applied to

the fetal thorax during the expulsion phase of delivery, reduces lung volume below functional residual capacity. In his conception, elastic recoil of the chest wall, once it is delivered from the birth canal, causes passive inflow of air. Our data show that a negative intra-pulmonary pressure is already established in advanced labour before the thorax enters the birth canal. It is also likely that the liquid absorption seen in the last 1 - 2 hours of labour (Brown *et al*, 1983) reduces intra-pulmonary pressure to levels below those seen in this study.

It is interesting to consider the extent to which the observed negative intrapulmonary pressure in labour would affect lung liquid secretion or reabsorption. Active reabsorption of liquid, driven by Na^+ transport in the last hours of labour, would progressively reduce lung volume, increase chest wall recoil, and result in an increasingly negative intra-pulmonary pressure. The increasingly negative intra-pulmonary pressure established during labour would, however, favour passive movement of liquid into the lung lumen and potentially counterbalance active absorptive mechanisms. Rather than reduce the luminal space to dryness, a balance would be achieved at which active reabsorption matched passive inflow of liquid, with no further net liquid movement.

5.5.4 Resistance in tracheal loop catheter

The reported compliance of the liquid-filled fetal lung is high, with values around 5 $ml.cmH_2O^{-1}.kg^{-1}$ (Dickson and Harding, 1991), or slightly less according to our results. Any obstruction in the tracheal loop could therefore give rise to a large volume increase in the lung while causing only a slow build-up of pressure that would act to overcome the obstruction. In an attempt to assess whether an obstruction was present in the tracheal loop, we measured intra-pulmonary pressure at the start of each experiment. Two potential

sources of error should be considered in this measurement. First, all intra-pulmonary pressure values were referenced to amniotic fluid pressure measured through an open-ended catheter. If the tip of this catheter did not lie in a liquid pool that was contiguous within the amniotic sac, as could occur with low amniotic fluid levels near term, amniotic pressure might not be correctly determined. Second, the measurement of intra-pulmonary pressure was taken from the caudal part of the loop at the start of the experiment, and if an obstruction existed along this tube we might have underestimated intra-pulmonary pressure. In retrospect, we should have determined intra-pulmonary pressure after eliminating any obstructing plug by vigorous aspiration and flushing of the tubing. Even so, our results show that in some cases the tracheal loop did indeed raise pressure in the lung, with some values in the 4 - 5 cm H_2O range, considerably above the average of 2 - 3 cm H_2O (Vilos and Liggins, 1982; Dickson and Harding, 1991).

5.5.5 Mechanisms underlying the decline in V_L during labour

Having established that V_L declines over the course of the last days of gestation, and that the process begins well before the start of labour, and in the absence of active liquid reabsorption, the key issue is what brings it about. We have earlier reported that fetal activity is altered in labour, with a cessation of breathing and the appearance of long-lasting contractions of the diaphragm, together with activity resembling coughing (Berger *et al*, 1986). During the course of the present study, long-lasting and simultaneous contractions of thoracic, abdominal and diaphragm muscles were observed during labour. As we have suggested (Berger *et al*, 1998), such fetal activity could conceivably expel liquid from the fetal lung. What stimulates the fetus to initiate expulsive trunk muscle contractions is unknown. Levels of cortisol and many other circulating hormones are known to rise during labour, and one or more of these agents could trigger the fetus to alter

its behaviour. In agreement with an earlier study (Berger *et al*, 1986), we found no significant hypoxia, hypercapnia or acidosis during labour at the time fetal lung liquid volume had declined substantially, demonstrating that fetal asphyxia is not involved.

Earlier work demonstrates that Na^+ reabsorption, activated by the adrenaline surge of labour, begins to clear liquid from the lung in the last 1 - 2 hours before birth (Brown *et al*, 1983). The extent to which this mechanism reduces lung liquid volume cannot be decided from our study, but it could be responsible for the decline in volume from the 13.8 $ml.kg^{-1}$ we observed in advanced labour to the level of 6.8 $ml.kg^{-1}$ reported at the end of labour (Berger *et al*, 1998). It is difficult to envisage how active expulsive efforts by the fetus could achieve this final stage in the decline in lung liquid volume, since it occurs in a lung that is already well below functional residual capacity and in conditions of substantial negative intra-pleural pressure. Although the fall in lung liquid volume that may be attributable to reabsorption is only a small fraction of the total liquid clearance before delivery, previous work indicates that it would have a substantial beneficial effect on postnatal gas exchange (Berger *et al*, 1996; Berger *et al*, 2000). Once switched on in labour, the Na^+ absorptive mechanism continues to play a crucial role in pulmonary function throughout postnatal life (Ramsden *et al*, 1992). Its importance is underlined by the respiratory distress experienced by newborn guinea pigs in which pulmonary Na^+ channels are blocked with amiloride (O'Brodovich *et al*, 1990a). Perhaps more tellingly, when the Na^+ channel is rendered defective genetically, newborn mice die of respiratory failure within 8 to 40 hours (Hummler *et al*, 1996).

5.5.6 Clinical significance

Our elucidation of the timetable with which lung liquid volume and secretion decline

before term delivery emphasises the importance of the last days of gestation in adapting the fetus for postnatal life. The benefits of labour to the newborn are already well illustrated by the higher incidence of respiratory compromise ("wet lung") in human infants delivered by caesarean section with absent (12.4%) or reduced (5.6%) labour, compared with a much lower incidence (0.6%) in infants that deliver vaginally (Hales *et al*, 1993). The mechanisms that adapt the lung for postnatal life can now be seen to include a prolonged and gradual clearance of lung liquid beginning well before the onset of labour, together with an acceleration of clearance once labour is established. The respiratory hazard that elective caesarean delivery represents may, therefore, not simply be attributable to the absence of labour, but also to the newborn missing out on a process that clears liquid from the lung over a period of days leading up to labour.

CHAPTER SIX

CYCLIC LUNG EXPANSION REDUCES LUNG LIQUID SECRETION IN NEAR TERM FETAL SHEEP

CYCLIC LUNG EXPANSION REDUCES LUNG LIQUID SECRETION IN NEAR TERM FETAL SHEEP

6.1 ABSTRACT

For gas exchange to be established postnatally the liquid filling the lung must be rapidly cleared. Active Na^+ transport from alveolar to interstitial space appears to play an essential role in early neonatal adaptation with severe respiratory distress and death occurring at birth if Na^+-channels are blocked by amiloride or absent as a result of gene manipulation. Several hormones such as adrenaline and AVP have been shown to induce absorption during labour, but their effect vanishes when their levels fall rapidly after birth. It therefore remains unclear what mechanisms maintain absorption in the newborn and adult lung.

Several authors suggested that stretching of the pulmonary epithelium during the first breaths at birth may induce absorption of liquid by active Na^+ transport, although this trigger mechanism remains disputed.

We have tested whether lung expansion, mimicking the effects of breathing at birth, triggers the amiloride-blockable Na^+-absorption mechanism in vivo, using unanaesthetised fetal sheep near term.

Expansion of the fetal lungs to a level close to total lung capacity caused a significant fall in secretion rate from 3.9 ± 0.4 ml.kg^{-1}.h^{-1} to 0.9 ± 0.6 ml.kg^{-1}.h^{-1}, and in 3 of the 8 animals studied absorption was initiated.

Addition of 10^{-4} M amiloride to the lung liquid to block Na^+ absorption whilst the lungs

remained cycled at the same high volume, did not lead to a restoration of secretion to its initial level; indeed secretion rate remained depressed (1.0 ± 0.9 ml.kg^{-1}.h^{-1}). We conclude that in the near term fetal sheep cyclic lung expansion between RV and TLC reduces lung liquid secretion rate by a mechanism which is not amiloride sensitive and we speculate that it may result from an increase in pore size with passive liquid flow.

6.2 Introduction

During fetal life the lung is a secretory organ that maintains the future airspace in a distended state as a result of Cl^- secretion across the epithelium (Olver and Strang, 1974). By contrast, in postnatal life pulmonary gas exchange requires the airspace to be kept essentially dry. Recent work demonstrates that most of the liquid filling the fetal lung is cleared by the end of labour (see Chapter 5; Berger *et al*, 1998; Pfister *et al*, submitted for publication). However, in order to be permanently adapted for air-breathing, the lung must be transformed from a secretory organ to one which is characterised by liquid absorption. This requirement is met in the postnatal lung by trans-epithelial Na^+ absorption (Basset *et al*, 1987b; Effros *et al*, 1987; Berthiaume *et al*, 1988) and the required transformation at birth may simply be achieved by shifting from Cl^- secretion to Na^+ absorption (Olver *et al*, 1986). The mechanism by which the lung acquires and maintains this new absorbing capability for the rest of adult life is of particular interest, as it may give insights into the pathophysiology of some frequent respiratory illnesses in newborns and adults. To date, however, the trigger mechanisms for this vital transformation at birth have been only partly elucidated. What is known is that reduced secretion or absorption of lung liquid can be triggered in the late gestation fetal lung by adrenaline (Brown *et al*, 1983; Olver *et al*, 1986), cAMP (Walters *et al*, 1990) and vasopressin (AVP) (Perks and Cassin, 1982; Cassin and Perks, 1993). However, at birth these hormones can act only as temporary

triggers for absorption, since their circulating levels fall soon after delivery, and their effect is known to disappear rapidly when their levels decline.

Lung liquid secretion rate has also been shown to be sensitive to the degree to which the lung is expanded (Egan, 1976; Perks and Cassin, 1985b; Ramsden and Neil, 1993; Vejlstrup *et al*, 1994; Kojwang and Perks, 1996). The effect of lung expansion in reducing secretion rate (J_v), or even inducing absorption, was first described in fetal sheep (Egan, 1976), and later confirmed in fetal goats (Perks and Cassin, 1985b). In these studies, the extent of the effect was reported to be directly related to the degree of expansion; furthermore, the effect appeared to continue for as long as distension was maintained. These findings suggested that the maintenance of lung distension during postnatal air breathing may induce Na^+ absorption and may also maintain the lung epithelium in an absorptive mode throughout postnatal life.

While studies of several different designs have demonstrated a reduction in secretion, or the induction of absorption after lung expansion, many of the preparations used to date are likely to be physiologically unstable. For example, studies have been performed in exteriorised lungs (Garrad-Nelson and Perks, 1996), in exteriorised and anaesthetised fetuses (Egan *et al*, 1975; Perks and Cassin, 1985), in postnatal and adult animals in which the lungs were artificially perfused (Ramsden and Neil, 1993) or in partially-ventilated lungs (Egan *et al*, 1976; Vejlstrup *et al*, 1994). Some of these preparations have a potential for epithelial hypoxia which clearly reduces secretion rate by itself, as was first demonstrated by blockade of the respiratory chain with cyanide (Olver and Strang, 1974). This study was designed to investigate whether lung distension, carried out under conditions that create minimal disturbance to the fetus, and at a gestational age near term

to ensure that the lung is mature, causes a reduction in lung liquid secretion rate or induces absorption of liquid from the lung lumen. Further, we tested whether the effect of expansion is mediated via activation of an amiloride-sensitive Na^+ transport mechanism.

6.3 Materials and Methods

6.3.1 Surgery

Ethical approval for all experiments was obtained from the Monash University Standing Committee on Ethics in Animal Experimentation.

The fetuses of pregnant Border-Leicester ewes were instrumented at 132 days gestation (G132; term = G147). Anaesthesia was induced with iv thiopental sodium (1-2 g: Pentothal, Abbott, Australia) and maintained with 66% N_2O: 1-2% Halothane: 32-33% O_2. The maternal abdomen was opened in the midline and the fetal head was delivered through a uterine incision. The carotid artery was catheterized non-occlusively and two wide-bore silastic catheters (ID 3 mm, OD 6 mm; Sil-Med Corporation, Taunton, Massachusetts) were introduced through a single tracheostomy, with one catheter directed rostrally so that the tip lay 2 cm or more from the larynx, while the other catheter was directed caudally towards the lungs. The two free catheter ends were connected to form a liquid-filled loop. A liquid-filled catheter for measuring amniotic fluid pressure was secured to the skin of the fetal neck. The fetus was returned to the uterus which was then filled with warm saline to restore fluid lost during surgery. The uterus and abdominal wall were sutured and all catheters were tunnelled to the ewe's flank before exiting through a small incision. All animals received post-operative analgesia (50 mg Finadyne, Schering-Plough, Australia) and daily intramuscular antibiotic treatment (500 mg procaine penicillin, 500 mg dihydrostreptomycin). Animals were allowed to recover for 8 to 10 days before

observations commenced.

6.3.2 Experimental procedure

Period 1: Cycling between residual volume and functional residual capacity

At G142 the limb of the tracheal loop directed towards the lung was connected to a temperature-controlled (40°C) glass burette closed at its top by a rubber bung penetrated by an 18-gauge needle with an attached millipore filter. This arrangement allowed liquid to be drained from, and re-instilled into, the lung under sterile conditions while evaporative water loss was minimised. First, lung liquid was drained by lowering the burette 15 - 20 cm below the estimated fetal position until no further liquid left the lung by gravity, leaving a volume of liquid in the lungs corresponding to residual volume (RV). Then, approximately 50 - 100 ml of the drained lung liquid was transferred to a sterile container and a known amount of RISA (10 - 20 μC) was mixed into it after having taken a sample for determining background. A further sample of the labelled lung liquid was removed before reintroducing the precisely weighed remaining volume to the burette. A large quantity of RISA was used in this study to ensure that an accurate volume determination could be made after a large volume of artificial lung liquid was added during Period 2 of the protocol (see below). The tracer was mixed with lung liquid by repeated cycles of drainage and re-instillation using a maximum hydrostatic pressure of approximately ± 15 - 20 cm H_2O.

The magnitude of the volume of liquid that cycled into and out of the lung (ΔV_L) was the variable stimulus between experimental periods. During Period 1, ΔV_L was the difference between RV and resting lung liquid volume, which represents functional residual capacity (FRC) in the fetus. Each cycle lasted approximately 1 - 2 minutes (Figure 6-1). After at

least 30 min of cycling, during which the tracer was thoroughly mixed into lung liquid, between 12 and 15 samples (0.5 ml) were taken at 5 - 10 min intervals for radioactivity measurements. Full details on the preparation and validation of radio-iodinated serum albumin (RISA) for use as an impermeant tracer are given elsewhere (see Chapter 4).

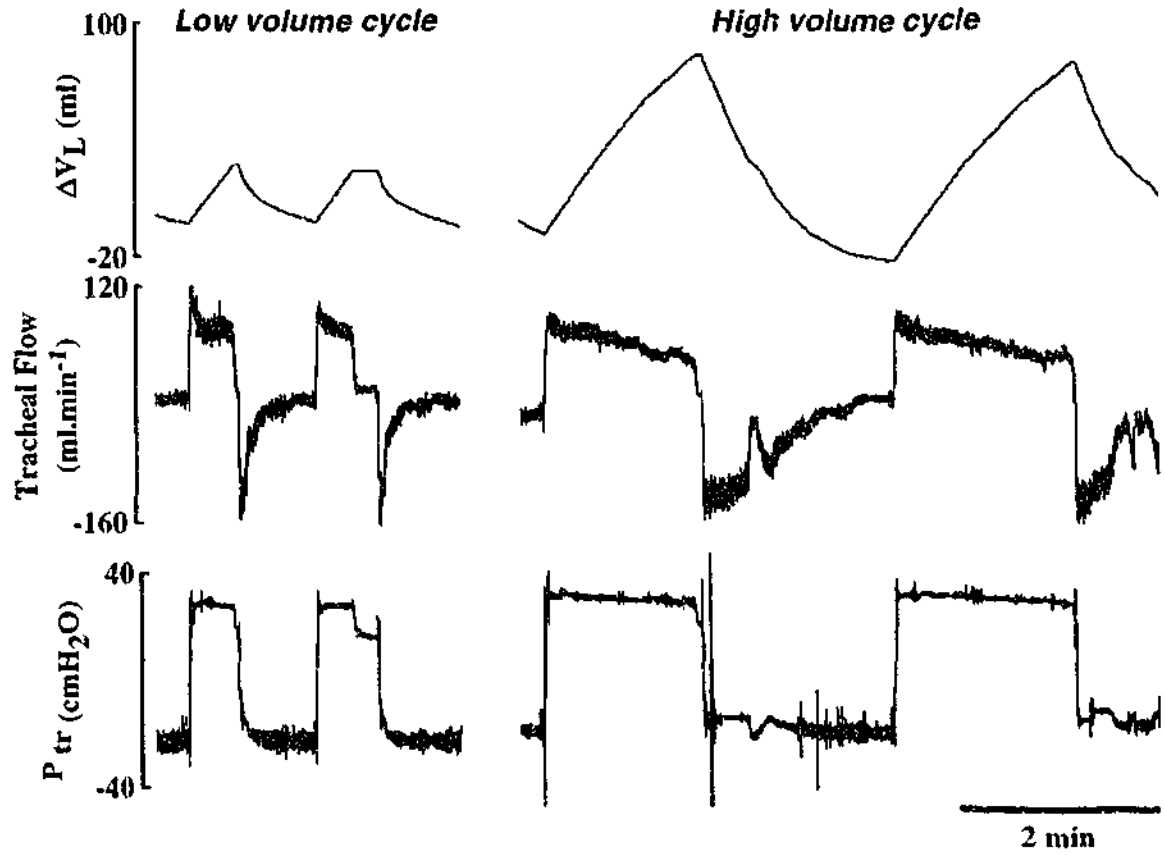

Figure 6-1. *Example of the volume cycling protocol obtained during a pilot experiment, with cycles ranging from RV to FRC in the left hand panel similar to cycles obtained during Period 1 of the protocol, and from RV to TLC in the right hand panel similar to cycles during Periods 2 and 3. Volume was obtained by integration of the flow signal measured with an ultrasonic probe on the lung side of the tracheal loop.*

Period 2: Cycling between residual volume and total lung capacity

To expand the fetal lungs up to a volume close to total lung capacity (TLC), approximately 30 $ml.kg^{-1}$ of liquid was added to the burette at the end of Period 1. To achieve this goal we assumed a fetal weight of 4 kg and therefore added 120 ml of warm (40°C) artificial lung liquid. In Table 6-1 we report the actual expansion volumes based on body weight obtained at post-mortem. Cycles in Period 2 ranged from RV to TLC and were about twice as long as during Period 1, in the range of 2 - 4 minutes. After at least 30 min of this new regime a further 12 - 15 samples (each 0.5 ml) were taken at 5 - 10 min intervals whilst cycling continued.

Period 3: Na^+ channel blockade

We then assessed whether any effect of lung expansion on secretion involved the amiloride-sensitive Na^+ channel. To do so, we added amiloride (amiloride hypochloride, Sigma, St. Louis, USA) in 5 ml of injectable water to lung liquid to make up a final concentration of approximately 10^{-4} M in lung liquid; this concentration has been found to block Na^+ transport via the amiloride-sensitive Na^+ channel (Benos, 1982; Olver *et al*, 1986). ΔV_L remained unchanged from Period 2 (RV to TLC), and after 30 min of cycling a further 12 - 15 samples (each 0.5 ml) were taken at 5 - 10 min intervals.

At the end of the experiment the animal was killed painlessly with an overdose of phenobarbitone sodium (Lethabarb, Virbac, Peakhurst, NSW, Australia), the fetal weight taken and a post-mortem examination was performed.

6.3.3 Lung liquid volume and secretion rate

Concentration of RISA was measured on a gamma counter (1282 Compugamma, LKB,

Wallac) with a window set between 20 and 100 KeV. Lung liquid volume and secretion rates were calculated according to established techniques (Brown *et al*, 1983). This involved calculating secretion rates (J_v) as the slope of lung liquid volume against time using the least squares method. Lung liquid volume at the time of interruption of the tracheal loop ($V_{Lt=0}$) was obtained by extrapolation. Residual volume (RV) was assumed to remain constant and was calculated as the difference between the lung liquid volume determined by indicator dilution at the start of the experiment ($V_{Lt=0}$) and the volume of liquid that was drained at experiment onset. As a result of small change in cycle maximum during the course of each experiment, due to differences between lung liquid secretion and the volume of liquid removed during sampling, we report a mean value, calculated half-way through each experimental period ($V_{L,max}$).

6.3.4 Condition of pulmonary epithelium

Integrity of the alveolar membrane during the 3 experimental periods was assessed by measuring plasma radioactivity before using RISA and at the end of the third experimental period, and by monitoring lung liquid electrolytes (K^+, HCO_3^-) before and at the end of each experimental Period.

6.3.5 Data analysis and presentation

Differences between secretion rates of each period in each experiment were compared by analysis of variance of slopes. Summarised volumes and secretion rates for all animals were compared with repeated-measures ANOVA and Scheffé's post-hoc test.

Values are given as mean ± SEM. Differences were considered significant when $P < 0.05$.

6.4 RESULTS

All 8 fetuses were studied at G141 or G142 (weight 4.7 ± 0.3 kg) and were in good condition as shown by arterial blood gases and acid-base status during each experimental period (Table 6-1). Surgical preparation, macroscopic lung conditior. and general condition were normal at post-mortem carried out immediately after completion of the experiment. One fetus was excluded from the study because labour became evident during the experiment. Inclusion or exclusion of this animal did not alter the significance of the results presented.

***Table 6-1**. Lung liquid volume, secretion rate, haemoglobin and blood gases at experiment onset (T_o) and for each of the three experimental periods. Lung liquid volume (V_L) at experiment onset is extrapolated to time 0, whereas lung volume during each experimental period is the mean volume measured during that period. Blood gases were obtained from samples taken at the end of each period.*

	T_0	Period 1	Period 2	Period 3
V_L (ml.kg^{-1})	31.9 ± 2.1	34.6 ± 1.5	67.0 ± 3.0	67.1 ± 2.6
J_v (ml.kg^{-1}.h^{-1})		3.9 ± 0.4	0.9 ± 0.6	1.0 ± 0.9
Hb (g.l^{-1})	13.3 ± 0.8	12.9 ± 0.8	13.0 ± 0.8	13.3 ± 0.7
pHa	7.32 ± 0.02	7.32 ± 0.03	7.30 ± 0.03	7.30 ± 0.02
PaO_2, mmHg	22.3 ± 1.0	23.9 ± 1.3	24.2 ± 1.8	23.7 ± 1.4
$PaCO_2$, mmHg	55.0 ± 0.7	54.3 ± 1.6	54.4 ± 1.4	55.2 ± 1.4

Values are given as mean ± SEM.

6.4.1 Effect of lung expansion on secretion rate

An example of the low and high volume cycles imposed upon the fetal lung was recorded in a preliminary experiment, as illustrated in Figure 6-1. For the animals comprising this study, during Period 1 the volume cycle had a minimum value of 10.9 ± 1.4 ml.kg^{-1} and a maximum of 34.6 ± 1.5 ml.kg^{-1}. The cycle maximum (V_{Lmax}) was then roughly doubled during Periods 2 and 3, to 67.0 ± 3.0 ml.kg^{-1} and 68.6 ± 2.8 ml.kg^{-1} respectively.

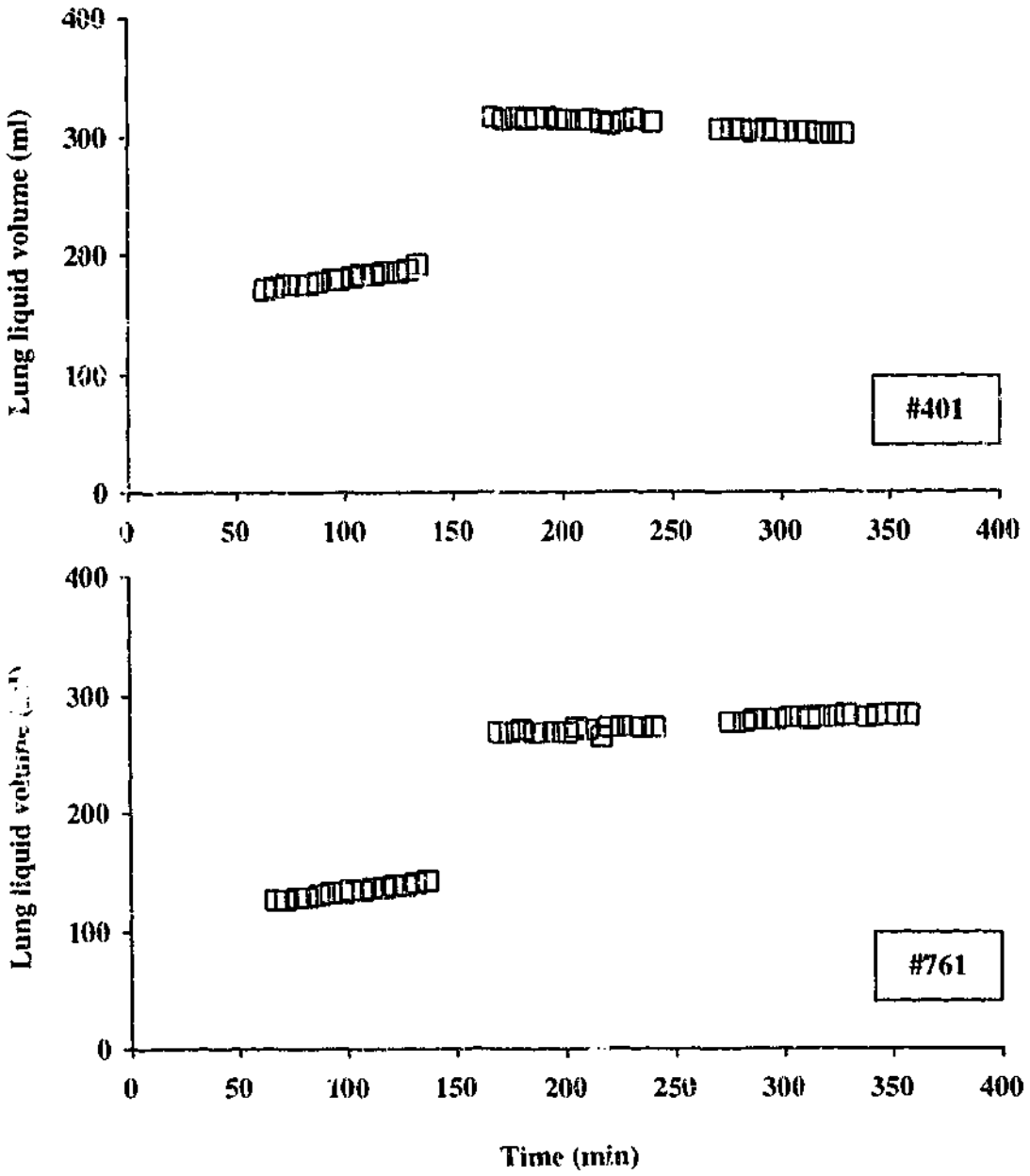

Figure 6-2. Typical experiments on two animals, one where the increased cycle volume (Period 2) triggered absorption and the second where the same stimulus reduced secretion but did not initiate absorption. Amiloride had no effect in both (Period 3), as was the case in the majority of experiments.

The changes in lung liquid volumes with time during the course of the experiment, are shown in Figure 6-2 for the two responses observed to expansion; that is, decreased secretion and absorption. In all but two animals, in which secretion remained unchanged, there was a decrease of secretion rate in Period 2, with absorption being initiated in 3 of the animals. For the group as a whole, the mean lung volume ($V_{Lt=0}$) and secretion rate at study onset were 31.9 ± 2.1 ml.kg^{-1} and 3.9 ± 0.4 ml.kg^{-1}.h^{-1} respectively.

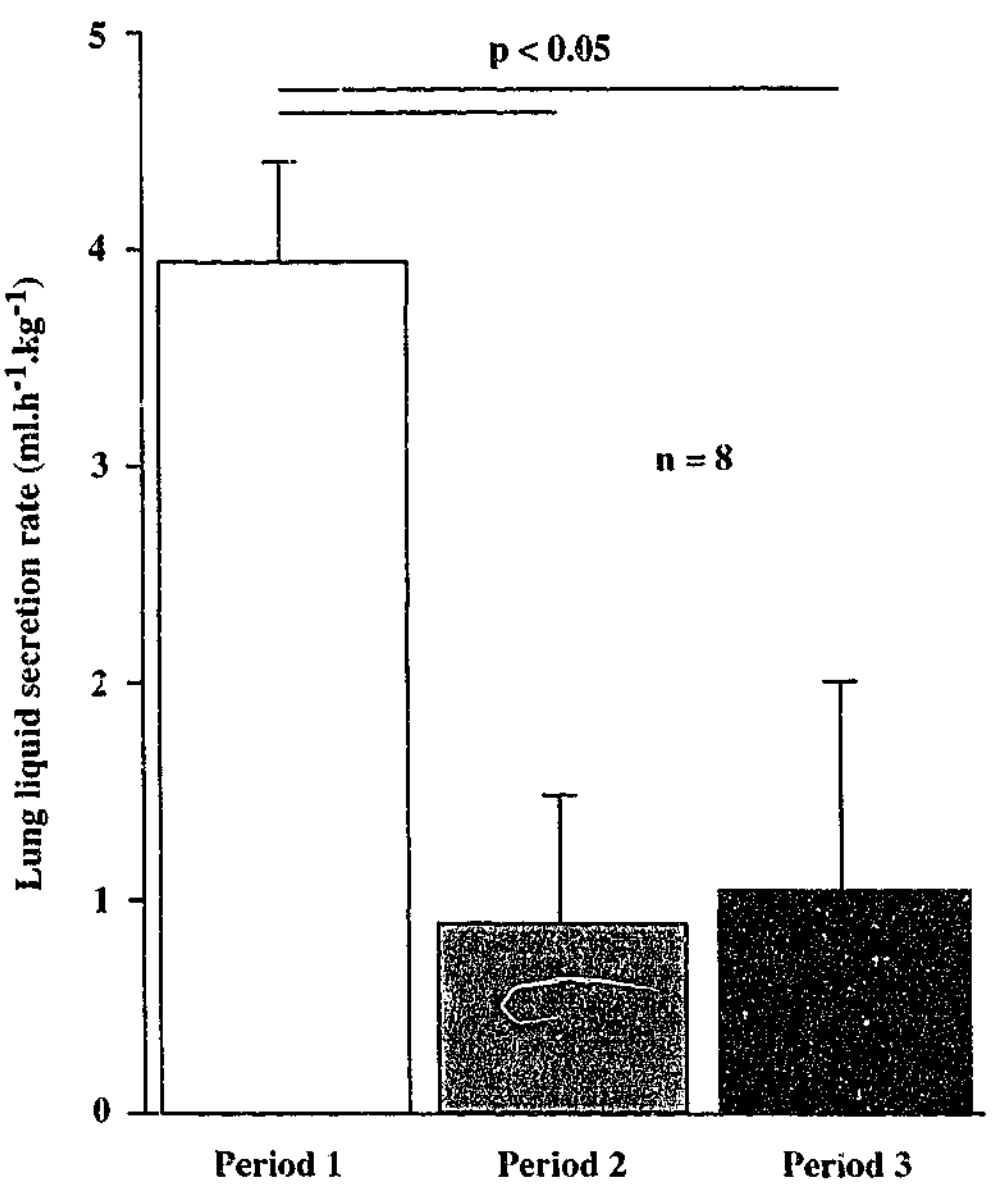

Figure 6-3. *Effect of cyclic lung expansion on lung liquid secretion rate. Period 1 is control with low cycling volume, Period 2 after increasing cycle volume by approximately 30 ml.kg^{-1} and Period 3 after addition of amiloride 10^{-4} M at high cycling volume.*

When the lungs were subjected to cyclic expansion in Period 2, secretion rate dropped significantly to 0.9 ± 0.6 ml.kg^{-1}.h^{-1} (Figure 6-3). Combining the data for Period 1 and 2, there was a significant relationship between for secretion rate and ΔV_L (Figure 6-4) and for secretion rate and V_{Lmax}. On the basis of the calculated regressions, reversal of secretion would be expected to occur at a ΔV_L of 66.4 ml.kg^{-1} or at a V_{Lmax} of 77.6 ml.kg^{-1}.

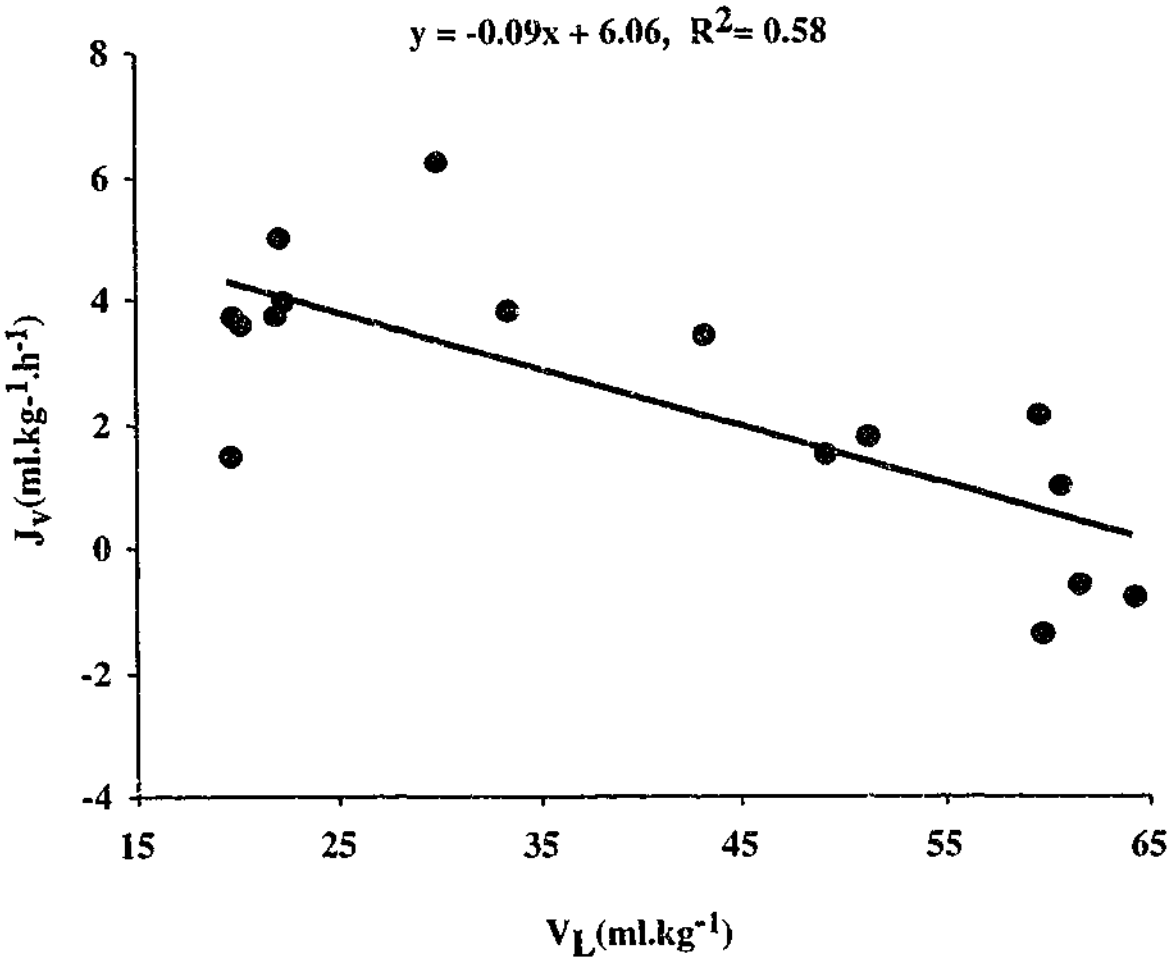

Figure 6-4. *Relationship between cycle volume (ΔV_L) and secretion rate (J_v) during low and high volume cycles (Periods 1 and 2).*

6.4.2 *Na^+ channel blockade with Amiloride*

Based on the lung liquid volumes determined by indicator dilution, the concentration of amiloride to which the pulmonary epithelium was exposed in these experiments ranged from 9.9×10^{-5} to 3.6×10^{-4} M, with a mean of 1.7×10^{-4}M. In the group as a whole, amiloride treatment had no effect on secretion rate which remained at 1.0 ± 0.9 ml.kg^{-1}.h^{-1} during Period 3. In one fetus (#661), where lung expansion induced absorption, amiloride reduced absorption in Period 3 but did not cause a return to secretion (Table 6-2). Another fetus (#181), which did not respond to cyclic expansion in Period 2, responded to amiloride with an increase in secretion rate (Table 6-2).

Table 6-2. *Individual secretion rates (ml.kg^{-1}.h^{-1}) during the three study periods. Secretion rates between study periods were compared for each animal by analysis of variance for multiple slopes and by Student's t-test. Differences were considered significant when $p < 0.05$. ΔV_{Lmax} in ml.kg^{-1} is the difference between the peak volume to which the lung was exposed during cycles in Period 1 and Period 2.*

Experiment #	Period 1	<->	Period 2	<->	Period 3	ΔV_{Lmax}
641	3.7	$p < 0.05$	1.8	$p < 0.05$	0.1	29.5
661	6.2	$p < 0.05$	-1.4	$p < 0.05$	-0.6	29.7
181	3.6	ns	3.4	$p < 0.05$	7.1	31.8
401	3.8	$p < 0.05$	-0.8	ns	-1.2	30.8
711	1.5	ns	1.5	ns	1.7	29.4
6.1C	3.9	$p < 0.05$	-0.6	$p < 0.05$	-2.0	39.1
20.1B	5.0	$p < 0.05$	2.1	ns	1.6	37.5
761	3.7	$p < 0.05$	1.0	ns	1.4	40.8
Average	**3.9**	**$p < 0.05$**	**0.9**	**ns**	**1.0**	**32.4**
SEM	**0.4**		**0.6**		**0.9**	**1.9**

6.4.3 Condition of pulmonary epithelium

The radioactivity measured in fetal plasma at the end of experimental Period 3 amounted to 0.55 ± 0.16% of the radioactivity level of the first lung liquid sample. Changes were noted in the concentrations of K^+ and HCO_3^- in lung liquid (Table 6-3). K^+ values did not change significantly between Periods 1 and 3, but there was a trend from 9.1 ± 0.8 $mmol.l^{-1}$ in Period 1 to 7.8 ± 0.7 $mmol.l^{-1}$ and 6.9 ± 0.7 $mmol.l^{-1}$ in Periods 2 and 3 respectively. HCO_3^- did not change from experiment onset to the end of Period 1, but rose significantly from 1.9 ± 0.2 $mmol.l^{-1}$ before the onset of the experiment, to 6.6 ± 1.4 $mmol.l^{-1}$ and 9.0 ± 1.8 $mmol.l^{-1}$ in Periods 2 and 3 respectively. As can be seen from examining Table 6-2 and Table 6-3, the biggest changes in K^+ and HCO_3^- occurred in those fetuses in which J_v changed most between Period 1 and Period 2.

Table 6-3. *Individual K^+ and HCO_3^- in lung liquid samples ($mmol.l^{-1}$) at experiment onset (T_0) and during each experimental period (mean of 4 values): the low volume period (P1), the high volume period (P2) and the high volume period with Amiloride (P3).*

	K^+				HCO_3^-			
exp #	**T_0**	**P1**	**P2**	**P3**	**T_0**	**P1**	**P2**	**P3**
641	10.6	8.9	9.3	8.6	1.2	2.9	3.3	5.8
661	7.5	8.1	5.8	5.2	1.6	2.0	12.8	14.1
181	8.0	8.8	8.7	7.3	1.5	1.5	2.5	2.0
401	8.3	8.3	6.1	5.0	1.4	3.1	7.8	15.8
711	10.5	11.0	9.9	8.4	2.0	2.0	2.5	3.3
6.1C	7.1	7.4	5.5	5.0	2.7	3.6	11.4	12.1
20.1B	6.8	7.0	6.4	5.3	2.5	2.8	5.9	7.4
761	13.6	14.7	10.4	10.1	2.4	2.8	6.6	11.4
Average	**9.1**	**9.3**	**7.8**	**6.9**	**1.9**	**2.6**	**6.6**	**9.0**
SEM	**0.8**	**0.9**	**0.7**	**0.7**	**0.2**	**0.2**	**1.4**	**1.8**

6.5 DISCUSSION

The principal finding of this study is that when the lung of the late-gestation sheep fetus is subjected to cyclic expansion with liquid, from a minimum approximating residual volume to a level close to total lung capacity, the rate of secretion of lung liquid is reduced. Indeed, in 3 fetuses lung expansion of this magnitude caused absorption. Since secretion rate was found to be strongly correlated both with cycle maximum (V_{Lmax}) and cycle volume (ΔV_L), the effect on secretion may be caused either by the degree of stretch or by the peak pressure imposed by expansion. Our results also show that the expansion-induced reduction in secretion, or initiation of absorption, is not blocked by amiloride, suggesting that it is either not mediated by epithelial Na^+ channels or, if Na^+ channels are involved, they are of an amiloride-resistant type.

6.5.1 Critique of the method

There were two key requirements in our protocol. First, it was essential that tissue hypoxia be avoided, since blockade of the respiratory chain of the lung epithelium has been shown to reduce secretion (Olver and Strang, 1974), as does fetal hypoxaemia (Wallace *et al*, 1996a). In our experimental design, lung volume exceeded FRC for half of each cycle in Periods 2 and 3. In the high volume phase of each cycle, V_{Lmax} rose to approximately 70 $ml.kg^{-1}$, a degree of inflation that has been shown to reduce lung perfusion (Walker *et al*, 1988). Thus, for a portion of the high volume cycles, it is possible that tissue hypoxia might have developed. However, reduction of lung volume to less than FRC leads to increased pulmonary perfusion (Walker *et al*, 1988), so the overall effect on lung tissue oxygenation of the volume cycles in Periods 2 and 3 is likely to have been minor. The second requirement is that there be no mechanical damage to the epithelium during lung expansion. Such damage appears unlikely in our experiments, since large expansions were

imposed for only short periods and the pressure during peak expansion was no greater than 20 cmH_2O. In addition, epithelial damage resulting from volo- or baro-trauma is unlikely as we measured only a trivial increase in serum radioactivity due to RISA leakage, and this was approximately equal to that measured in an earlier study which did not involve high volume expansion (see Chapter 4). In this earlier study, the highest radioactivity found in the thyroid was about 15 times higher than in the fetal blood. In late gestation the thyroid weighs at least 20 times less than the circulating blood volume, indicating there was only a small loss of tracer from the lung compartment. In addition, the earlier study suggested that leakage may have occurred as a result of free ^{125}I label crossing the pulmonary epithelium rather than the RISA molecule as a whole. Furthermore, in preliminary experiments using prolonged periods of high volume cycling similar to those employed in this study, the C^{14}-mannitol diffusion rate did not significantly change from control levels.

As we sought to examine whether lung expansion is a key mechanism behind the permanent transformation of the lung epithelium from secretion to absorption, it was important to begin with a normal starting volume. Lung liquid volume (31.9 ± 2.1 $ml.kg^{-1}$) and secretion rate (3.9 ± 0.4 $ml.kg^{-1}.h^{-1}$) obtained at G142 at the start of the experiment were comparable to other values in late gestation obtained with the RISA technique (Normand *et al*, 1971; Olver and Strang, 1974; Dickson *et al*, 1986; Dickson and Harding, 1989; Berger *et al*, 1996), a technique we have shown to produce reliable results (see Chapter 4). Accordingly we consider the resting lung liquid volume obtained in this study to be normal. Our lung liquid volumes are considerably less than the values obtained with Blue Dextran as the indicator, which are typically greater than 40 $ml.kg^{-1}$ in late gestation (Lines *et al*, 1997) and as high as 50 $ml.kg^{-1}$ (Harding and Hooper, 1996). As already discussed (see Chapter 4), the extremely high values of lung liquid volume reported in

these studies are likely to be explained by the observation that Blue Dextran binds to the lung in late gestation, making it highly likely that this tracer overestimates lung liquid volume at this time.

Contrasting with some of the previous studies which imposed a constant level of expansion on the lung (Egan, 1976; Vejlstrup *et al*, 1994), in this study the expansion stimulus applied had a cyclic pattern to mimic the effect of breathing. Others have also examined the effect of cyclic expansion on lung liquid secretion, but cycle volumes were poorly quantified (Perks and Cassin, 1985b) or V_{Lmax} only, but not ΔV_L, was modified (Garrad-Nelson and Perks, 1996). The cycle minimum (V_{Lmin}) we used remained constant during the whole experiment, and was measured at experiment onset to be 10.9 ± 1.4 ml.kg^{-1}, not far from the 6.8 ml.kg^{-1} measured in the fetal lung at the end of labour (Berger *et al*, 1998). The mean cycle maximum was modified during the different experimental periods, being 34.6 ± 1.5 ml.kg^{-1} during Period 1 and approximately double that value during Periods 2 and 3.

6.5.2 Effect of lung expansion

This is the first in vivo study of chronically-instrumented fetuses to examine the effect of cyclic lung expansion on lung liquid secretion. Cyclic expansion clearly reduced secretion rate in the group as a whole and, in 3 out of 8 animals studied, secretion was converted to absorption, suggestive of an active process. Overall, however, secretion continued in the group and the decline in secretion did not appear to result from activation of the amiloride-sensitive Na^+ transport mechanism. The simple regression between cycling volume and secretion rate suggests that cycling volumes larger than 70 ml.kg^{-1} would have reversed secretion. This is at the high end of the range used in our study, perhaps accounting for

absorption being observed in only 3 fetuses. It is also likely to lie outside the range of the cyclic changes in lung volume that occur during normal postnatal breathing, suggesting that this mechanism plays little role in converting the lung into an absorptive organ after birth.

Perks (Perks and Cassin, 1985b) reported that constant expansion of the lung by 50% or more of its control volume induced absorption in fetal goats. The values of 60 $ml.kg^{-1}$ obtained in the present study represents about a 100% increase in cycle maximum ($V_{T,max}$) and this would suggest that continuous expansion had a larger effect compared to our cyclic expansion. However the resting lung liquid volumes reported in the earlier study were highly variable (Perks and Cassin, 1985b), ranging from 26.1 to 54.6 $ml.kg^{-1}$, and when expansion is recalculated in terms of $ml.kg^{-1}$ body weight, the greatest reduction in secretion rate paradoxically occurred for the smallest expansion. There is, however, reason to be concerned about the method of volume determination in that study (Perks and Cassin, 1985b), since they used Blue Dextran, which introduces very large errors into lung liquid volume estimates (Chapter 4).

6.5.3 Mechanisms for modified secretion rate

Adrenaline and AVP both reduce secretion or induce absorption in the fetus near term, and this effect has been shown in a number of studies to be mediated by the amiloride-sensitive Na^+ channel (Olver *et al*, 1986; Ramsden *et al*, 1992; Cassin and Perks, 1993; Hooper *et al*, 1993b). By contrast, studies examining the mechanisms responsible for the absorption that occurs in response to lung expansion have produced conflicting findings. In isolated fetal guinea-pig lungs, expansion is reported to induce absorption of liquid from the lung lumen, an effect blocked by amiloride; however, after amiloride the lung did not return to

secretion (Garrad-Nelson and Perks, 1996). In the fetal guinea-pig, therefore, expansion appears to inhibit secretion by some unknown mechanism, and to promote absorption via amiloride-blockable Na^+ channels. In 36 - 60 day-old lambs, in which the lungs were blood-perfused in situ, an enhanced Na^+ absorption was observed when the lungs were expanded (Ramsden and Neil, 1993), and this effect was amiloride-sensitive. However absorption continued at a reduced rate after amiloride treatment, indicating that absorption results from at least one other mechanism in the postnatal lamb lung. By contrast, amiloride has been reported to arrest absorption in undistended lungs in adult rabbits, but to have no effect on absorption when the lungs are distended (Vejlstrup *et al*, 1994).

If the only mechanism responsible for absorption in the postnatal lung were to be the amiloride-sensitive Na^+ transport mechanism, blockade with amiloride should result in a return to secretion at a rate similar to that in the fetus, independent of the age of the lungs distended. The results of a number of studies are clearly not consistent with this prediction in that secretion did not return after amiloride treatment, or if it did it was at a rate less than that in the fetus (Ramsden *et al*, 1992; Ramsden and Neil, 1993; Vejlstrup *et al*, 1994; Garrad-Nelson and Perks, 1996). Thus some mechanism other than the amiloride-sensitive Na^+ channel must be postulated. Based on potential differences, and on electrolyte gradients demonstrating selective passage of several electrolytes, the fetal alveolar epithelium has been described as "leaky" (Olver and Strang, 1974). Later studies confirmed the accuracy of this description by demonstrating an increase in epithelial pore size after inflation of the fetal or adult sheep lung, an increase which was shown to occur naturally between fetal and newborn life (Egan *et al*, 1975; Egan, 1976). Our results also support the existence of an amiloride-resistant pathway for regulation of lung liquid secretion in the mature fetus, and the dose/response relation we found between cycling

volume and secretion rate is in accordance with the existence of pores or non-specific channels which modify permeability to electrolytes when the lung is stretched. Pores offer the potential for liquid clearance to be driven by the difference in colloid-osmotic forces between lung liquid and blood, a difference which approximates 20 mmHg (Boston *et al*, 1968), and this mechanism may result in faster clearance than would occur by active Na^+ transport. An alternative, that the hydrostatic pressure between lung lumen and interstitium drives water through epithelial pores appears unlikely, because mean intra-luminal pressure is not high in our experiments, since mean lung volume was less than resting volume (FRC) in Period 1, and approximated FRC in Periods 2 and 3 (see Figure 6-1).

In addition to the pore hypothesis, our results may be explained by the presence of a transport mechanism involving a Na^+ channel with low amiloride affinity, just as Na^+ channels of different amiloride affinity coexist in fetal rat epithelial cells (Basset *et al*, 1987a; Matalon *et al*, 1993). This hypothesis is also consistent with reports that there are few amiloride-sensitive Na^+ channels in fetal life, whereas these channels, as reflected by their mRNA levels, increase enormously after birth (O'Brodovich *et al*, 1993; Voilley *et al*, 1994). Whether pore or channel, it is unlikely that expansion permanently activates the mechanism of sustained absorption throughout adult life, as pore size has been shown to increase only transiently in the perinatal period (Egan *et al*, 1975) and as the post-natally predominant amiloride-sensitive Na^+ channel (O'Brodovich *et al*, 1993; Voilley *et al*, 1994) was not activated by expansion in our experiments.

In conclusion we have shown that cyclic lung expansion in fetal sheep near term reduces secretion significantly and in some fetuses induces absorption. Since this effect is not reversed by blockade of the amiloride-sensitive Na^+ channel, this pathway is not

implicated in the effect, contrasting with the absorption brought about by adrenaline and AVP. We propose that lung expansion in the near-term fetus achieves the arrest of secretion, or initiates absorption, by increasing the size of pores in the epithelium through which a colloid-osmotic pressure gradient causes lung liquid to shift into the interstitium and plasma. Alternatively, it is possible that expansion achieves its effect via non-amiloride sensitive Na^+ transport.

Whatever the mechanism, the activation of an absorptive mechanism by lung expansion suggests that the mechanical consequences of breathing at birth may play a role in the removal of liquid from the airspace. However, the very large volume cycles required to elicit a decline in secretion, or in a small number of fetuses an initiation of absorption, indicate that expansion per se does not play the main role in generating lung liquid clearance across the pulmonary epithelium. Nevertheless, our findings indicate that expansion can affect trans-epithelial liquid clearance, and it is possible that this effect is more substantial with air than with liquid. Thus, the results presented in this Chapter demonstrate that in addition to an amiloride-sensitive transport mechanism, clearance of liquid from the lung may be assisted by a second pathway. This biologically crucial process of lung liquid clearance at birth may therefore be ensured by at least two different mechanisms, one that is amiloride-sensitive and the other amiloride-resistant.

CHAPTER SEVEN

SYNTHESIS

SYNTHESIS

7.1 SUMMARY

At the time this experimental program began, there already existed a large body of evidence showing that an active secretory process (Olver and Strang, 1974) leads to the fetal lung being filled with liquid to a level approximating functional residual capacity. Further, it had been shown that distension of the lung with liquid during fetal life plays an essential role in the normal development of the lung (Alcorn *et al*, 1977). It was also known that the large volume of liquid filling the lung of the late gestation fetus had been cleared from the lumen within the first six hours after term delivery (Bland *et al*, 1982). There was evidence that at least a part of this clearance occurred as a result of active Na+ transport from the lung lumen, beginning within the last one or two hours of labour (Brown *et al*, 1983). A single study had also reported evidence that lung liquid volume began to decline in advance of labour (Dickson *et al*, 1986), perhaps as a result of a decline in the rate of lung liquid secretion prior to the start of labour (Kitterman *et al*, 1979).

Collectively, the available evidence provided a strong basis for considering that the mechanisms underlying lung liquid secretion and absorption are controlled in a highly adaptive fashion, with a fetal period characterised by the maintenance of a distending volume of liquid that promotes lung growth and differentiation, and a perinatal period in which the lung is converted into a relatively dry organ to facilitate gas exchange. This scenario was in keeping with epidemiological evidence that showed infants delivered electively by caesarean section before onset of labour have a greater incidence of respiratory morbidity than infants delivered after onset of labour, and these babies in turn have greater respiratory morbidity than babies delivered vaginally (Hales *et al*, 1993).

However, the Thesis began at a time when there was dispute as to whether lung liquid volume does indeed start to decline in advance of labour. In a series of reports, evidence was presented that the volume of liquid distending the fetal lung continues to rise over the final weeks of gestation until the start of labour, when it reaches an average level more than 50% higher than FRC (Hooper and Harding, 1995; Harding and Hooper, 1996; Lines *et al*, 1997). These reports were surprising in that they were difficult to reconcile with epidemiological evidence showing a reduced respiratory morbidity with age in infants delivered electively by caesarean section (Robert *et al*, 1976). In addition, the levels of lung liquid volume in the late gestation fetal sheep were considerably higher than those reported from other laboratories.

Disagreement about the timing of the decline in lung liquid volume in the perinatal period is an important issue for resolution for two reasons. First, the timing of the decline may suggest possible mechanisms that could be tested experimentally. Second, it may alter clinical decisions about the timing of an elective caesarean delivery, particularly in those situations where the surfactant system can be considered to be mature and the predominant postnatal morbidity is likely to be "wet lung". Clearly, if lung liquid volume were to rise in the final days of gestation, it would be best to perform an operative delivery earlier rather than later; conversely, if the process that gives rise to a decline in lung liquid volume is set in train before establishment of labour, it would be best to delay an operative delivery until as close to term as possible. If, however, the volume of liquid in the lungs begins to decline only once the fetus is in labour, an operative delivery should be delayed until some time within labour; the question that would then need to be answered is how much labour does it take to reduce lung liquid volume to a level that maximally assists the gas exchange capacity of the newborn lung?

The first task undertaken in this Thesis was to make a critical evaluation of the methods used in studies examining whether there is a pre-labour decline or rise in lung liquid volume. Almost all studies have used indicator dilution to estimate lung liquid volume, but the study reporting a decline before labour used radio-iodinated serum albumen (RISA) as the volume tracer (Dickson *et al*, 1986), while those reporting a continuous increase in lung liquid volume right up to the day before labour used Blue Dextran (BD) as the tracer (Hooper and Harding, 1995; Harding and Hooper, 1996; Lines *et al*, 1997). As detailed in Chapter 4, RISA satisfies the assumptions of the indicator-dilution technique in the late gestation fetal sheep lung, manifesting very little binding with the pulmonary epithelium and very little loss across the epithelium. Thus estimates of lung liquid volume obtained with RISA can be accepted. By contrast, those derived from BD are unsound. We have demonstrated that BD contravenes the assumption that the volume tracer remains free in the liquid whose volume is being estimated; instead, BD bound in considerable quantity to the lung epithelium. As a result, use of BD as volume tracer in the fetal sheep lung would lead to an overestimation of volume, and our finding of increased binding near term indicates that the volume overestimate would be greater in late gestation. A further complication in the use of BD is that the mixing technique used to distribute the tracer evenly throughout the lung causes the release of material from the pulmonary epithelium that has high absorbance in the same spectral range as that of BD. Since optical absorbance is the method by which the concentration of BD is measured, this feature of the fetal lung also renders the use of BD problematic.

Failure of BD to satisfy the requirements of the indicator dilution technique makes it reasonable to discount reports that lung liquid volume rises over the last 3 weeks of gestation in the fetal sheep. However, the claim that the volume of liquid in the fetal lung

falls before labour has also been criticised in that labour was not monitored adequately (Lines *et al*, 1997). Thus the fall reported by Dickson (Dickson *et al*, 1986) may have occurred within and not before labour. In view of the importance of establishing when lung liquid volume declines in the perinatal period, a longitudinal study was undertaken using RISA as the volume tracer. Particular attention was taken to record uterine activity and amniotic fluid pressure continuously near term in order to detect the onset of labour. This study represents the core of the Thesis and the results presented in Chapter 5 add weight to the finding that BD is likely to overestimate lung liquid volume, especially near term, in that there is no increase in volume over the last days of gestation when RISA is used as the volume tracer. Instead, the results confirm that there is a decline in lung liquid volume over the last 3 days of gestation, and they also show that the decline occurs in two phases. The first phase in the decline in lung liquid volume is gradual and occurs over a period of approximately 3 days leading up to labour, followed by an accelerated rate of decline that is restricted to labour.

A notable feature of the findings presented in Chapter 5 is that the reduction of lung liquid volume to 13.8 $ml.kg^{-1}$ does not come about through trans-alveolar absorption. It is therefore reasonable to conclude that lung liquid is cleared in the last days of gestation, and up to the stage of labour at which we terminated the study, via trans-tracheal expulsion of liquid. The mechanisms underlying this mode of clearance are currently unknown and require elucidation.

Trans-epithelial liquid clearance seems to mainly play a role in postnatal life. As lung liquid absorption was not observed throughout the period of study in other than in a single animal, it is tempting to speculate that absorption of liquid across the pulmonary

epithelium would have been observed had the experiment been allowed to continue. The present findings support earlier work (Brown *et al*, 1983) showing that absorption of liquid becomes an important means of removing liquid from the lung only very close to delivery, and, as other studies have shown, absorption then dominates lung water balance throughout the remainder of life. While it is known that a rise in circulating adrenaline levels, and perhaps AVP, near the end of labour initiate Na^+ transport across the pulmonary epithelium (Perks and Cassin, 1982; Brown *et al*, 1983), and thereby lead to reabsorption of lung liquid, it is still unclear what maintains the absorptive mechanism once the levels of these hormones decline soon after birth. The final study reported in this Thesis investigated the possible role of cyclic lung expansion or stretch, in promoting lung liquid absorption in the postnatal lung. The underlying hypothesis was that cyclic lung expansion, mimicking postnatal ventilation, triggers active and lasting liquid absorption. The findings lead to the rejection of this hypothesis, although expansion was found to reduce secretion. However, the mechanism by which this effect was achieved appeared to involve increased alveolar permeability rather than activation of the amiloride sensitive Na^+ channel. The conclusion from this study is that the effect of expansion may be of importance in passive clearance of liquid in the immediate postnatal period as an additional pathway to the well demonstrated active Na^+ transport mechanism.

7.2 Future Investigations

In further work it will be of great interest to investigate possible mechanisms that trigger active prenatal trans-tracheal liquid expulsion by the fetus. If possible this should be done in a model that does not involve the use of a laryngeal loop that imposes a significant resistance to lung liquid outflow.

Despite possible prevention strategies, incomplete or delayed lung liquid clearance will continue to account for a considerable proportion of neonatal respiratory morbidity. Postnatal respiratory disease may result from insufficient clearance before birth but also from insufficient or delayed postnatal liquid transport, as very clearly demonstrated by pharmacological blockade or knock out of the alveolar Na^+ channel (O'Brodovich *et al*, 1990a; Hummler *et al*, 1996). As, according to our findings, this trans-alveolar liquid clearance is activated only in the very last stage of labour, or in early postnatal life, an early neonatal intervention in the form of stimulation of the Na^+ channel appears a promising prospect. The amiloride sensitive Na^+ transport can be activated by adrenergic stimulation and other pharmacological agents in its downstream intra-cellular cascade, as for example cAMP. Agents known for activation of the Na^+ channel should be tested in early postnatal interventions for their beneficial effect on lung adaptation as well as for treatment of respiratory diseases of the newborn.

7.3 Conclusions

The findings documented in this Thesis underline the importance of late gestation and labour in relation to adapting the lung for postnatal gas exchange. The evidence presented demonstrates that approximately 30% of the liquid present in the lung of the late gestation fetal sheep is cleared in the 3 days leading up to labour. Between the time that labour begins, and the point at which labour is advanced and pushing begins, there is a decline of a similar size that takes the volume of liquid in the lung to approximately 14 $ml.kg^{-1}$. Based upon two earlier published studies, a further decline in lung liquid volume then occurs, reducing lung liquid volume to approximately 7 $ml.kg^{-1}$ before the fetus delivers. The Thesis provided no insights into the mechanism underlying the final step in the clearance process, but it seems likely that adrenaline-induced stimulation of Na^+ transport

may be responsible. Whatever the cause of the final stage in lung liquid clearance, the results of this Thesis favour postponement of operative delivery until as close to term as possible in order that the fetus derive the benefit of the gradual decline in lung liquid volume observed before labour, and the more rapid decline associated with labour. On the basis of the work presented here, reducing the steadily increasing rate of planned caesarean section delivery before term presents a key preventive intervention to reduce neonatal respiratory morbidity. From the fetal point of view, labour is a process to seek rather than avoid.

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