THE EFFECT OF METABOLIC INHIBITORS, PIRETANIDE, SOMATOSTATIN AND INSULIN ON FLUID SECRETION BY IN VITRO FETAL LUNGS FROM GUINEA PIGS (Cavia porcellus).

By

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This study introduced the isolated lungs of fetal guinea pigs as a new preparation for the investigation of prenatal lung physiology. It established their basic properties by the use of metabolic inhibitors and the Cl⁻ transport inhibitor, piretanide. In addition, the effects of two naturally occurring peptides, somatostatin and insulin were investigated. It also extended studies to a species which has received no investigation by modern methods.

The fetal lungs of fetuses from near term guinea pigs (54 to 67 days gestation) were used. The total volume of lung fluid was measured during three hours by dye-dilution of Blue Dextran 2000. In the second hour the lungs were exposed to one of the substances under investigation.

Untreated fetal lungs continued to secrete in vitro for three hours. The average rate of secretion during the first hour (2.31 ± 0.17 ml/Kg per h; n=104) is comparable to secretion rates reported in chronically catheterized fetal lambs. The combined data of the control groups showed a steady increase in lung fluid volume throughout the three hours of experiment. Sodium iodoacetate (10⁻³M and 10⁻⁴M), an inhibitor of the glycolytic pathway, reduced lung fluid secretion. Sodium fluoride, an alternative inhibitor of glycolysis, also reduced secretion. The possible importance of the aerobic metabolic pathways was tested by the use of NaCN (10⁻³M). The results were more variable than those from the glycolytic inhibitors, and suggest that NaCN slightly reduces the secretion rate. Sodium azide was tested as an alternative inhibitor of the aerobic metabolic pathway with similar results to those from NaCN, confirming that the oxidative pathway has some influence on lung secretion, although it does not appear as important as the glycolytic system. Dinitrophenol was tested as an alternative inhibitor of oxidative processes. The results suggest that
dinitrophenol not only abolishes secretion but also produces reabsorption. Piretanide, a loop diuretic capable of inhibiting Na⁺/K⁺/Cl⁻ co-transport, reduced lung fluid secretion rates at both 10⁻⁷M and 10⁻⁸M. Somatostatin, a natural inhibitor of Cl⁻ secretion in some tissues, reduced secretion at both 10⁻⁵M and 10⁻⁶M (no significant effect at 10⁻⁷M). Insulin is known to influence the maturity of fetal lungs, and to stimulate Na⁺ transport in some tissues; Na⁺ transport is probably involved in reabsorption. The results showed that insulin at 10⁻⁶ - 10⁻⁷M reduced secretion by the isolated lung. This study suggests that the in vitro fetal lung could be a useful tool for future study.
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INTRODUCTION

The possibility that the fetal lung contains liquid was apparently considered as early as 1787 (Wislow: quoted by Preyer 1885, in Olver & Strang 1974). The trachea and bronchi, during intra-uterine life, are filled with liquid which for many years was assumed to be aspirated amniotic fluid (Adams et al. 1963a).

The presence of lung liquid in the fetal lungs is almost certainly important for lung development (Liggins 1984). It must determine the shape and volume of peripheral lung units, as well as the growth and form of vascular, epithelial and connective tissue components; fetal lung liquid also seems to contribute significantly to the formation of amniotic fluid (Strang 1977a).

ORIGIN OF FETAL LUNG LIQUID

The first experimental study that dealt with lung fluid was that of Leclard (1815, in William et al. 1913) who clamped the neck of a living fetus, and on opening the trachea found a fluid analogous to amniotic fluid. Subsequent investigations by Geyl (1880, in William et al. 1913) and Preyer (1885, in William et al. 1913) verified Leclard's findings.

Addison and How (1913-14, in DeSa 1969) concluded that the liquid of the "trachea, bronchi and lungs" resembles amniotic fluid but was not necessarily inspired from the amniotic cavity, and that the pulmonary liquid was absorbed rapidly at birth, when the air-lung is established (DeSa 1969, Olver & Strang 1974).

It was not until 1941 that the origin of this fluid was seriously questioned. Potter and Bohlender (1941 and Avery & Mead 1959, in Adams et al. 1963a), presented the first compelling evidence that the fetal pulmonary fluid was produced locally in the lung. Later, Whitehead et al. (1942, in Carmel et al. 1965) supported this possibility, and suggested that the fluid
did not originate from the amniotic space, but flowed outward from the lung, and therefore prevented amniotic debris from occluding future airways. At this point the perennial question of amniotic fluid aspiration in utero had been turned round 180 degrees. Soon afterwards, Jost and Policard (1948) produced clear evidence that the liquid inside the lung was formed within the lung. In their study, the fetus was decapitated in utero, and the trachea ligated; this produced a dilatation of the alveoli, presumably due to accumulation of fluid. However, because of the severity of the process of decapitation the significance of the results remained dubious (Carmel et al. 1965).

Reynolds (1953) was the first to attempt to determine the origin of the lung fluid in live intact fetuses (Reynolds 1953, Avery 1968). During his experiments, significant secretion of fluid occurred. However he wrongly attributed the secretory activity to the nasopharynx and buccal cavities, rather than to the lungs themselves (Adams et al. 1963a, Olver & Strang 1974).

The possibility that secretions from the nasopharyngeal and buccal cavities, as well as the lung itself contribute to the amniotic fluid has been considered by Macafee (1950, in Adams et al. 1963b), by Bevis (1953, in Adams et al. 1963b) and more recently by Setnikar and others (Setnikar et al. 1959, Villee 1960, in Adams et al. 1963b, Bein & Scott 1960, in Adams et al. 1963b). Setnikar and his co-workers performed experiments on goats and guinea pigs during the last third of pregnancy. Their findings demonstrated that the fetal lung produces fluid, and this supported the original ideas of Whitehead et al. (1942, in Carmel et al. 1965).

The same result was obtained by Carmel et al. (1965), who performed the study under near-physiological conditions and showed that a fluid is produced in the lung, and that normal lung development occurs in the
absence of amniotic fluid aspiration. This was done by ligating the trachea of rabbit fetuses several days prior to term and allowing the fetuses to remain in utero until term, at which time they were delivered by Caesarean section, and the lung examined (Carmel et al. 1965, Strang 1977a). Carmel suggested that the fluid is produced in the fetal lung against a hydrostatic pressure. With no escape channel for the fluid, the lung would be forced to expand more and more as the fluid was produced. This process would continue at least until the hydrostatic pressure in the potential air spaces counterbalanced the forces producing the fluid.

Because lung fluid is actively secreted, it is unlikely that amniotic fluid would enter the lungs except during fetal breathing movements. However, there is evidence that strongly suggests that amniotic fluid aspiration does occur with stress or hypoxia. In contrast, in normal conditions the lung fluid must move into the pharynx and then pass through the nose and mouth into the amniotic cavity, or is swallowed (Carmel et al. 1965).

Adams and his colleagues published three reports (Adams et al. 1963a, Adams et al. 1963b, Adams et al. 1971) that also claimed the pulmonary origin of fetal lung fluid and suggested that small volumes of fetal pulmonary fluid are released periodically from the lung, and either swallowed or enter the amniotic cavity. They indicated that the pulmonary fluid may be a product of ultrafiltration, with selective secretion or reabsorption.

**NATURE OF FETAL LUNG FLUID**

The physico-chemical features of lung fluid in the last third of pregnancy are very similar to those found in interstitial fluid (Hanon et al. 1955, in Setnikar et al. 1959), particularly in animals in which the allantoic sac is still present during this period, e.g., goats and sheep
(Alexander et al. 1958, in Setnikar et al. 1959). It was possible that the lung fluid, like interstitial fluid, could be produced by a process of ultrafiltration. This production would require an organ with a large surface area supplied by many capillaries (Meschia 1955, in Setnikar et al. 1959). Two organs present such properties: kidneys and lungs. The lung seems to be a source of amniotic fluid, perhaps of particular importance in those animals in which the urethra becomes patent only late in fetal life. In animals in which the urethra becomes patent earlier (e.g. man and guinea pig), the kidney also contributes to production of amniotic fluid (Setnikar et al. 1959).

However, more direct evidence for an active secretion of lung liquid, rather than ultrafiltration, was provided by Strang (1967, in Strang 1977a), and his evidence was based on measurements of the flux ratios of cations and anions between lung fluid and plasma.

DIFFERENCES BETWEEN THE LUNG FLUID, AMNIOTIC FLUID AND PLASMA

The lung fluid itself was colorless, turbid and initially sometimes viscid. Adams, Moss & Fagan (1963) showed that lung liquid differed in composition from both amniotic liquid and plasma in almost every respect (Adams et al. 1963b, Olver & Strang 1974). This also supported the origin of the fluid in the lung itself, rather than in the amniotic cavity. Adamson, Boyd, Platt and Strang (1969) obtained the following values for plasma and lung liquid concentrations (protein in g/100 ml.; ions in m-equiv/kg H2O):

<table>
<thead>
<tr>
<th></th>
<th>[Protein]</th>
<th>[Na+]</th>
<th>[K+]</th>
<th>[Cl-]</th>
<th>[HCO3-]</th>
<th>pH</th>
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<td>Plasma</td>
<td>6.27</td>
<td>150</td>
<td>4.8</td>
<td>107</td>
<td>24.0</td>
<td>7.34</td>
</tr>
<tr>
<td>Lung liquid</td>
<td>0.03</td>
<td>150</td>
<td>6.3</td>
<td>157</td>
<td>2.8</td>
<td>6.27</td>
</tr>
</tbody>
</table>

The other differences between lung liquid and plasma, noted by Adamson et al. (1969), were a very low protein concentration (0.027
g/100ml), a low Ca$^{2+}$ level and apparently a lack of phosphates in the lung liquid. The low protein concentration was explained by Norman et al. (1970) in terms of the small dimensions of water-filled pores in alveolar walls which excluded the penetration of plasma proteins. The lower Ca$^{2+}$ in alveolar liquid may be explained in two possible ways: (1) by Ca$^{2+}$ binding to proteins in the plasma, or (2) by the low permeability of the alveolar epithelium to Ca$^{2+}$, in keeping with the large hydrated radius ($a \approx 4.1$ Å), and the narrow radius of pores in the alveolar walls (Normand et al. 1970). Similarly, the large hydrated radius of phosphates ($a \approx 4.1$-$4.5$ Å) could account for their low concentration in alveolar liquid (Olver & Strang 1974). Finally, a number of earlier workers were impressed by the high Cl$^-$/content of the lung liquid (Adams et al. 1971).

All these properties, together with the low total CO$_2$ content and low pH suggest that alveolar liquid is a special fluid elaborated by the fetal lung and that it is neither a simple ultrafiltrate nor a mixture of amniotic liquid with other secretions (Adamson et al. 1969, Humphreys et al. 1967, Strang 1977a, Olver & Strang 1974). No other body fluid has this composition. (Adamson 1969, Strang 1977a).

**SECRETION OF LUNG LIQUID: THE ACTIVE CHLORIDE TRANSPORT AND PASSIVE FLUX**

The lungs of the mature fetal lamb contain about 30 ml of lung liquid/kg body weight, and secrete liquid at a rate of between 3 and 5 ml/kg body weight per h (Olver & Strang 1974, Normand et al. 1971, Olver et al. 1981b). Thus a fetal lamb near term secretes a volume approaching half a liter every 24 hours. The liquid volume contained in the potential airspace appears to have an important role in normal lung growth; interference with lung volume, either by artificially withdrawing liquid or by preventing its escape on a long-term basis profoundly alters lung histology and tissue

The alveolar epithelium carries out two well-documented transport functions. During fetal life, the secretion of Cl- into the developing air space is the major driving force for the production of lung liquid (Strang 1974, in Gatzy 1983). In contrast, in the lung of the adult, surfactant and, presumably, small amounts of associated electrolyte solution are secreted by the epithelial cells onto the luminal surface (Mason et al. 1977, Gatzy 1983).

For a secretory organ to be capable of generating a chemical gradient, a barrier must be present to restrict molecular diffusion. Normand et al. (1971) showed that in the fetal lung this barrier resides in the pulmonary epithelium. In their experiments, the capillary endothelium of the fetal lung was found to present little barrier to molecular diffusion, whereas the pulmonary epithelium was relatively 'tight', completely restricting the movement of molecules larger than mannitol. Describing their results in terms of pore theory, the effective pore radius of the capillary endothelium was in excess of 11 nm, while that of the epithelium was only 0.6 nm. This may explain why lung liquid contains such a small concentration of protein (molecular radius of albumin is about 3.5 nm) (Walters & Ramsden 1985).

Several years ago Silva and co-workers (1977, in Gatzy 1983) proposed a scheme to explain Cl- secretion by the shark rectal gland. This model was later applied to other Cl- secreting and NaCl absorbing epithelia (Frizzell et al. 1979, in Gatzy 1983). The hypothesis depended on the active Na+ transport by the epithelium, allied to a recirculation of the Na+ ion (Figure 1). In Cl- secreting epithelia Na+ is assumed to be transported out of the epithelial cells across the basolateral membranes by a process that requires a Na+,K+, Mg-ATPase and is ouabain sensitive. Transepithelial Na+
transport results only if the apical (luminal) cell membranes are permeable to Na\(^+\). This permeability is selectively inhibited by amiloride. The Na\(^+\) and Cl\(^-\) enter the cells across the basolateral membranes by a neutral coupled process that is driven by the chemical Na\(^+\) gradient. Because the cell interior is electronegative with respect to interstitial (submucosal), and usually the luminal compartments, some of the accumulated Cl\(^-\) moves down the electrochemical gradient across apical membranes through a conductive path (Walters & Ramsden 1985).

The shark rectal gland appears to share similar mechanisms of Cl\(^-\) transport with a number of other epithelia, including the corneal epithelium (Nagel & Reinach 1980, Candia \textit{et al.} 1981, in Welsh 1987), large and small mammalian intestine (Fiel 1979, Frizzel & Heintze 1979), opercular epithelium of teleost (Degnan \textit{et al.} 1977, in Welsh 1987), gastric mucosa (Machen & McLenan 1980, in Welsh 1987) and the canine tracheal epithelium, which might be expected to show similarities to the fetal alveolar cells (Widdicombe \textit{et al.} 1979, Gatzi 1983).

Tracheal epithelium contains a Na\(^+\),K\(^+\)-ATPase (Westenfelder \textit{et al.} 1980, in Welsh 1987) that has been localized to the basolateral membrane by autoradiographic ouabain-binding (Widdicombe \textit{et al.} 1979, Welsh 1987).

When active Na\(^+\) transport by epithelial cells is inhibited by ouabain, or K\(^+\) removed from the submucosal bathing solution, the Na\(^+\) and thus Cl\(^-\) secretion is also inhibited (Al-Bazzaz & Al-Awqati 1979, Widdicombe \textit{et al.} 1979a, Widdicombe \textit{et al.} 1979b). Net transepithelial Na\(^+\) movement stops, the intracellular Na\(^+\) concentration rises, while cell K\(^+\) falls, intracellular negativity declines, and Cl\(^-\) enters. Increased cell Cl\(^-\) and Na\(^+\) would be expected to increase back flow through the coupled carrier, so that the unidirectional flux of Cl\(^-\) from lumen to interstitium might be expected to increase (Walters & Ramsden 1985).
The Na\textsuperscript{+},K\textsuperscript{+}-ATPase functions in the epithelium in a mode similar to that observed in other cells, extruding Na\textsuperscript{+} from the cell and accumulating K\textsuperscript{+}. As a result, the intracellular Na\textsuperscript{+} concentration is low and the K\textsuperscript{+} activity is high. K\textsuperscript{+} entering the cell on the Na\textsuperscript{+},K\textsuperscript{+} pump must be recycled across the basolateral membrane, since the rate of transepithelial K\textsuperscript{+} secretion is minimal (Welsh 1987).

Several observations suggest that the model of Cl\textsuperscript{−} secretion by surface epithelial cells or airway epithelia is compatible with the model discussed above (Figure 1).

The cell secretes Cl\textsuperscript{−} from the submucosal surface to the mucosal surface and absorbs Na\textsuperscript{+} in the opposite direction; both processes are electrogenic. The main features of the model are:

1). Cl\textsuperscript{−} enters the cell across the basolateral membrane via an electrically neutral NaCl cotransport process. Because Cl\textsuperscript{−} is coupled to Na\textsuperscript{+}, the movement of Na\textsuperscript{+} "down" a favorable electrochemical gradient across the basolateral membrane drives Cl\textsuperscript{−} "uphill" against its electrochemical gradient. Thereby, Cl\textsuperscript{−} is accumulated within the cell at an activity greater than predicted for electrochemical equilibrium with the energy provided indirectly by the Na\textsuperscript{+} electrochemical gradient.

2). Na\textsuperscript{+}, which enters the cell at the basolateral membrane coupled to Cl\textsuperscript{−}, exits back across the basolateral membrane via the Na\textsuperscript{+},K\textsuperscript{+} ATPase. This enzyme keeps intracellular Na\textsuperscript{+} activity low and thus supplies the nonconjugate energy for transepithelial Cl\textsuperscript{−} secretion. K\textsuperscript{+}, which enters the cell in exchange for Na\textsuperscript{+} on the Na\textsuperscript{+},K\textsuperscript{+} pump, exits passively across the K\textsuperscript{+} permeable basolateral membrane.

3). Cl\textsuperscript{−} leaves the cell passively, moving down a favorable electrochemical gradient across a Cl\textsuperscript{−} conductive apical cell membrane. Secretagogues appear to regulate the permeability of the apical membrane to
Figure 1. A cellular model of ion transport in Cl⁻ secreting epithelium.

Secretory: the cell secretes Cl⁻ from the submucosal surface (blood) to the mucosal surface (lumen) and absorbs Na⁺ in the opposite direction; both processes are electrogenic. In Cl⁻ secreting epithelia Na⁺ is assumed to be transported out of the epithelial cells across the basolateral membranes by a process that requires a Na⁺,K⁺,Mg-ATPase and is ouabain sensitive. Chloride accumulates beyond its electrochemical equilibrium inside the cell as a result of secondary active transport across the basolateral membrane via Na⁺,K⁺/2Cl⁻ co-transport system that is inhibitable by loop diuretics such as piretanide and bumetanide. Regulation of ion transport is achieved by second-messenger gating of apical chloride channels, which when open allow chloride to exit down its concentration gradient into the lumen.

Absorptive: in this model, the paracellular path is relatively impermeable to sodium, which now moves transcellularly. The entrance of Na⁺ through the apical membrane of the cell is selectively inhibited by amiloride.
CI\(^-\), thus controlling the rate of Cl\(^-\) secretion.

4). The apical membrane is also permeable to Na\(^+\) ions. Na\(^+\) may enter the cell passively at the apical membrane driven by a favorable electrochemical gradient.

5). Accordingly, during secretion, transepithelial current flow has several components. At the apical membrane, current is carried by conductive Cl\(^-\) exit and, to a lesser degree, Na\(^+\) entry, while at the basolateral membrane, current is carried by conductive K\(^+\) exit and Na\(^+\) exit via the Na\(^+,\) K\(^+\) pump.

When furosemide is added to the submucosal bathing solution it specifically inhibits Cl\(^-\) secretion but does not alter the rate of Na\(^+\) absorption (Ludens 1982, in Gatzy 1983, Welsh 1987). On the other hand, in the eye, exposure of the corneal surface to a concentration of amphotericin B that increases transepithelial Cl\(^-\) permeability abolishes the inhibition effects of loop diuretics on Cl\(^-\) secretion (Candia et al. 1981, in Gatzy 1983). Consequently, both coupled Na-Cl entry and passive Cl\(^-\) exit may be inhibited by loop diuretics (Gatzy 1983).

Cl\(^-\) is accumulated against an electrochemical gradient of 35 mV in the canine trachea. Possibly some intracellular mediator may regulate the Cl\(^-\) entry process. One reasonable candidate to regulate the Cl\(^-\) entry mechanism is intracellular cAMP, since it is known to increase with stimulation of secretion. Furthermore, cAMP is known to mediate electrically neutral Cl\(^-\) entry mechanisms in other epithelia, shark rectal gland, (Silva et al. 1977) and flounder intestine (Frizzell et al. 1979, in Welsh 1987). Cl\(^-\) accumulation is Na\(^+\) dependent; removing Na\(^+\) inhibits Cl\(^-\) entry at the basolateral membrane and allows Cl\(^-\) to distribute passively across a Cl\(^-\)-conductive apical membrane (Welsh 1987).

These observations, taken together, indicate that Cl\(^-\) is accumulated
across the basolateral membrane, against its electrochemical gradient, via a 
Na⁺ dependent, electrically neutral transport process that is inhibited by 
loop diuretics.

While these considerations provide compelling evidence for Na⁺
coupled Cl⁻ entry, the identity of the transport process is not known with 
certainty. There are several possible neutral Cl⁻ transport processes that 
might mediate Cl⁻ entry: (1) NaCl cotransport, (2) cotransport of 2 Cl⁻, 1 
Na⁺ and 1 K⁺, a process found in Ehrlich ascites cells (Geck et al. 1980, in 
Welsh 1987); or (3) parallel countertransport of Na⁺-H⁺ and Cl⁻-HCO₃⁻, 
proposed as the mechanism in the rabbit ileum (Liedtke & Hopfer 1982, in 
Welsh 1987). The third alternative seems unlikely since neither removing 
HCO₃⁻ from the bathing solution (Al-Bazzaz & Al-Awqui 1979) nor adding 
substituted stilbenes (which inhibit Cl⁻/HCO₃⁻ exchange in other cells) 
(Cabantchik & Rothstein 1974, in Welsh 1987) inhibits Cl⁻ secretion in 
tracheal epithelium (Welsh 1987).

Epinephrine stimulates Cl⁻ secretion in the tracheal epithelium 
secretagogues is an increase in the apical Cl⁻ permeability (Welsh 1987).

K⁺ entering the cell on the Na⁺,K⁺ pump must be recycled across the 
basolateral membrane, since the rate of transepithelial K⁺ secretion is 
minimal. Several observations indicate that K⁺ exits passively across a 
basolateral membrane that is predominantly K⁺ conductive (Welsh 1987). As 
the rate of Cl⁻ secretion increases, the rate of Na⁺ entry into the cell 
(via the Na⁺-coupled Cl⁻ entry process) will increase; as a result, the rate 
of Na⁺ extrusion (via the Na⁺,K⁺ pump) will increase. Given a constant pump 
stoichiometry, the rate of K⁺ entry into the cell will also increase. If K⁺ 
is to recycle back across the basolateral membrane, either the driving force 
for K⁺ must increase or the basolateral membrane K⁺ permeability must
increase (Welsh 1987).

Na⁺ absorption by tracheal epithelium is an electrogenic process. Mucosal amiloride inhibits Na⁺ absorption (Widdicombe & Welsh 1980). Although a variety of neurohumoral agents mediate the rate of Cl⁻ secretion, no endogenous substance has yet been found that stimulates the rate of Na⁺ absorption.

Although the details of the mechanisms of the formation of lung liquid are still not clear, the system probably operates on the same principles as other Cl⁻ secretory epithelia (Olver 1977).

**ABSORPTION OF THE LUNG FLUID**

Lung liquid continues to be secreted early in labour, but as delivery of the fetus approaches, secretion slows, and absorption of the lung liquid is observed when a presenting part (the forelimbs) appears at the vaginal outlet (Walters et al. 1978). The effect appears to be mediated by β-receptors, since it is blocked by propanolol, and a large concentration of noradrenaline has little effect, whereas isoprenaline is even more potent than adrenaline (Walters et al. 1978). According to Walters & Ramsden (1985) all of the slowing of secretion and the absorption of lung liquid observed during labour can be explained by the endogenous release of adrenaline by the fetus.

It is plain that much of the liquid present must be absorbed at the start of breathing. It is difficult to aspirate more than half of what is actually present through a tracheostomy, so it is unlikely that much fluid is expelled through the mouth during vaginal delivery (and even less would be expected to drain away after Caesarean section. From lung weight and lymph flow measurements, Humphreys et al. (1967) showed that much of the liquid is cleared by lymphatics from the interstitial spaces of the lung over a period of 5-6 h, but it seems likely that it must be displaced from
the alveolar spaces much more rapidly, otherwise efficient gas exchange would not be possible (Olver et al. 1974).

Olver & Strang (1974) found that arrest of lung liquid secretion, brought about by adding KCN to lung liquid, was followed by its absorption, but at such a rate that approximately 12 h would be needed to clear the lungs. The slow rate was attributed to the low permeability of the fetal alveolar walls to Cl\(^-\), the principal anion in the lung liquid (Egan et al. 1975).

The apparent anomaly here is that from what we know about alveolar permeability to Cl\(^-\), the slowest moving of the bulk solutes present in lung liquid, we would expect complete absorption to take in excess of 10 hours. However, Humphrey and co-workers (Humphreys et al. 1967) have shown that all the alveolar liquid is cleared from the lungs in 4-6 hours in newborn lambs (about 40% via-lymph and the rest directly into the circulation), and observations in humans and animals show that a functional residual capacity and efficient gas exchange are established within a few minutes of birth (Olver 1977).

Although there are probably, at the onset of breathing, hydrostatic as well as protein osmotic pressure gradients across the alveolar epithelium favouring absorption, it was concluded that, whatever their magnitude, the removal rate would be limited by the movement of Cl\(^-\) (and Na\(^+\)) into the interstitium (Egan et al. 1975). Rapid clearance could only be achieved at birth by an increase in pore size or increase in the epithelial area. Egan et al. (1976) have demonstrated that pore size increases some eightfold at birth, and they postulate that stretching of the epithelium facilitates the movement of small solutes, and hence water, between cells (Olver 1977).

**MECHANISMS OF LUNG LIQUID ABSORPTION**

Absorption of fetal lung liquid could be the result of several
different mechanisms. For example, the large protein osmotic difference between lung liquid and the blood plasma would be expected to produce passive absorption of liquid if the active transport of chloride into the luminal space were to be totally inhibited. In addition, an increase in permeability to ions, but not to protein, could also help fluid reabsorption. Indeed, a temporary increase in epithelial permeability of this type, presumably due to stretching of the alveolar surface, has been described in the first few hours of spontaneous breathing in neonatal lambs (Egan et al. 1975). It is possible that alterations in pulmonary blood flow could affect liquid movement across the pulmonary epithelium by an effect on the hydrostatic gradient. However, Olver and Strang demonstrated that changes in pulmonary vascular pressure sufficient to double lung lymph flow had no effect on the rate of lung liquid secretion (Olver & Strang 1974). Furthermore, adrenaline can induce rapid absorption of lung liquid in a dose that has no measurable effect on pulmonary blood flow, as detected by electromagnetic flow meters placed around the pulmonary artery. Also it is known that isoprenaline and adrenaline have opposite effects on the pulmonary vasculature but similar effects on lung liquid secretion (Cassin & Perks 1982).

Studies with the diuretic, amiloride, suggest that \( \beta \)-receptor stimulation of the fetal lung appears to stimulate sodium transport from lumen to plasma, an effect which is dependent on activation of sodium channels in the apical surface of the epithelial cells. This could be the result of configurational changes of pre-existing channels, or the insertion of preformed channels into the membrane. When an adrenaline infusion is stopped, the effect on the lung wears off rapidly and the epithelium begins to secrete again within minutes (Walters & Ramsden 1985).

Normal gas exchange in the mammalian lung requires virtually fluid
free airways and alveoli. It is likely that these air spaces are maintained in their relatively dry state by barriers that prevent the flow of fluid and solutes from the surrounding interstitial and vascular fluid into the alveoli and airways. Recent reviews (Staub 1974, in Crandall 1983) have discussed the factors that influence fluid balance between the air, interstitial and vascular spaces in the lung. Many fundamental studies have been performed on mammalian lungs, involving observations of the transfer of water and solutes between fluid-filled spaces, interstitial fluid (usually represented by lymph), and/or vascular fluid (Wangensteen et al. 1969, in Crandall 1983). It has been concluded that the epithelial barrier lining the air spaces of the adult lung is a major factor in preventing the movement of fluid and solutes into the alveoli (Taylor & Gaar 1970, in Crandall 1983). In addition the transfer of macromolecules across the alveolar epithelial barrier is reduced by the tight junctions (Scheeberger-Keeley & Karnovsky 1968, Scheeberger-Keeley & Karnovski 1971, in Crandall 1983).

If any justification is needed for presenting data from the fetal lung to those predominantly interested in air breathing, it is that study of the fetus may give us useful insight into alveolar fluid balance in the adult. Being naturally fluid-filled, the fetal lung lends itself to the in vivo study of transepithelial transport of solutes and water in a way that does not pertain after birth (Olver 1983). Fetal lung fluid is very low in bicarbonate, suggesting that acid secretion takes place (Adamson et al. 1969). Immediately after birth, the air-blood barrier becomes somewhat leaky, and then tight again (Egan et al. 1969, in Olver et al. 1981a), and active sodium transport out of the alveoli may be involved in clearance of lung fluid (Olver et al. 1981a, Crandall 1983).

The overview of the fetal lungs, and their fluid production, presented here suggests that a more sophisticated understanding of this
system is beginning to emerge. However, studies have been limited almost entirely to the fetal sheep, with a little investigation of the fetal goat, a few early studies on rabbits, and one investigation of the guinea pig. The results have been applied to human problems at birth, yet there is no evidence that all mammals operate in the same way. Indeed, ruminant mammals often have a markedly different physiology from other groups.

Therefore, it seemed important to extend research to other mammals, which could possibly resemble the human baby more closely than the sheep. The guinea pig is particularly useful because it carries unusually large fetuses for its size, and is more readily available in large numbers than the sheep. In addition, the size of the fetal lungs presents important new possibilities. While it is large enough to investigate by methods already established in fetal sheep, it is small enough to be maintained in vitro with the possibility of a continued secretion. Work with the acute goat and chronic sheep present particular difficulties in investigating detailed mechanisms of the secretion and control of the fetal lung. Many transport inhibitors and hormones may be destroyed rapidly in the intact animal, or lost through the placenta to the large reservoir of the mother, or act indirectly, and it is difficult to know the concentrations which act on the pulmonary epithelium. In addition, the inhibition may only act indirectly in the whole animal. More importantly, some drugs are toxic to the whole preparation—for example ouabain will stop the heart and kill the intact fetus. Some of these problems could be solved by tissue culture, and this has been attempted by some groups (Moss & Scarpelli 1979). However, this technique involves the loss of the organization of the lung. An in vitro preparation could allow the retention of the lung structure, and yet enable toxic drugs and inhibitors to be applied in known concentration to the serosal surface of the alveoli.
Therefore the work presented here extends studies to the guinea pig, which has only been investigated in one early study, which used relatively simple methods (Setnikar et al. 1959). It also investigates the basic properties of the lung secretion in the isolated preparation.
STATEMENT OF THE PROBLEM

The objective of this study was to introduce the isolated lungs of the fetal guinea pig as a new preparation for the study of the physiology of the lung prior to birth, and to establish its basic properties by the use of metabolic inhibitors (iodoacetate, fluoride, cyanide, azide, dinitrophenol) and of the Cl⁻ transport inhibitor (piretanide). In addition, the effects of two naturally occurring peptides, somatostatin and insulin were investigated. It also extends studies to a species which has received no investigation by modern methods.
METHODS AND MATERIALS

Near term guinea pigs, between 54 and 67 days gestation (normal term = 67 days) were used in the experiments carried out in this study. Whenever possible, ages were based on the previous delivery data, since guinea pigs go into oestrus and will mate immediately after parturition. In some cases, ages were estimated from fetal weights, using the average weight of each litter correlated to the number of fetuses (average litter size 4; range, 3-10), after the method of Ibsen (1928). The estimation was modified from age/weight data obtained from animals of known gestation age from our own stock. The guinea pigs were kept under optimal conditions in a vivarium, according to Canadian Guidelines for Animal Care. They were fed Purina rat chow and given free access to water.

SURGICAL PROCEDURES

The pregnant guinea pig was placed in a container which had a layer of ether-drenched shavings. The animal was left there for approximately ten minutes, until the blink reflex was extinguished. Then she was removed from the container and placed in a dissection tray.

A mid-line incision was made from the sternum through the abdomen to the bottom of the uterus. The uterus was then removed and the fetuses taken out individually, taking care that the amnion remained intact around the head. A single tight ligature was placed around the amnion and neck; this procedure effectively prevented the fetus from breathing. At this point the umbilical cord was cut, and each fetus was weighed rapidly and placed in a beaker containing Krebs-Henseleit saline at 37°C (see Appendix A).

A mid-line incision was made from the abdomen to the top of the neck of the fetus. The sternum was cut, with care not to damage the lungs. The rib cage was then opened to expose the lungs and trachea. The lungs and trachea were rinsed repeatedly with warm Krebs-Henseleit saline to keep them
moist and to prevent any accumulation of blood during dissection.

Two ligatures were passed under the trachea; the first was used to tie off the trachea as far rostrally as possible, the second to secure a cannula. The cannula was inserted through a slit placed below the upper ligature. The rostral end of the cannula was connected to a 3-way valve with a 1 cc glass tuberculin syringe. The cannula and the 3-way valve had previously been filled with warmed Krebs-Henseleit saline, taking care to remove any bubbles from the system. The 3-way valve allowed the fluid to be withdrawn and returned to the lung. In addition, fluid could be passed into the third cup of the stop-cock, and this allowed the addition of dye or the withdrawal of samples.

To remove the lungs together with the cannula system, the trachea was cut above the cannula. A slight tension was put on the trachea to allow the connective tissue to be cut beneath the preparation. The preparation was then separated from the body, and the heart was removed. Throughout the dissection, the lungs were rinsed with Krebs-Henseleit saline to keep the organ warm and moist. The lungs were then placed in a 50 ml container with Krebs-Henseleit saline bubbled with 95% O₂/5% CO₂, and kept at a constant temperature of 37° C in a water bath (Figure 2).

**EXPERIMENTAL PROCEDURES**

1). *Basis of the method*

The rate of lung liquid secretion was measured by a dye dilution technique, using Blue Dextran 2000, and based on the methods of Normand et al. (1971), and Perks & Cassin (1985b).

2). *Methods*

After placing the lung preparation in the 50 ml container of Krebs-Henseleit saline, lung fluid was withdrawn into the syringe and a 10 microlitre sample of the lung fluid was taken, as a blank, at zero time. 0.1
Figure 2. Apparatus for the maintenance of the isolated fetal lung.
ml of freshly dissolved Blue Dextran 2000 (50 mg/ml of 0.9% NaCl; Pharmacia, Uppsala Sweden) was mixed thoroughly into the remaining lung fluid and reinfused into the lung. At 5 minute intervals, lung fluid was gently mixed by withdrawing fluid and injecting it back into the lung 3 times with the tuberculin syringe. The mixing of the dye also helped to open up any pockets of the lung which the dye had not reached. Additional mixing was provided by the agitation of the preparation by the bubbles of the oxygen supply.

The lung preparation was allowed to equilibrate for 30 minutes in saline solution; the outer saline was replaced at 15 and 30 minutes, to remove any blood or other contaminants released by the dissection procedure, and to renew necessary materials.

Samples were taken every 10 minutes by drawing the lung fluid into the tuberculin syringe (after mixing) and then closing off the valve to the lung. The fluid could then be injected into the top part of the 3-way valve, where a sample could be taken by means of the 10 microlitre fixed volume syringe. Samples were then placed into 0.25 ml plastic microcentrifuge tubes and diluted 20 times with 0.19 ml of distilled water, using a fixed volume syringe. The samples were then mixed by putting them on a vortex mixer (Vortex Genie Fisher scientific) for five seconds. The samples were then centrifuged at approximately 1500 rpm for 10 minutes (Model 1221, International Equipment Co.)

The samples from the first hour after the equilibration period gave the basic rate of secretion of the lung preparation (control period). All samples were estimated for Blue Dextran 2000 by spectrophotometer (model 250, Gillford Instruments; used at 620 nm with 0.10 ml microcells). Readings on the spectrophotometer were taken nine times per sample, and then averaged.

In the second hour the lung was transferred to a bath which
contained one of the various substances used in this study. The test materials used were: Insulin $10^{-6}$ M, Insulin $10^{-7}$ M, Piretanide $10^{-8}$ M, Piretanide $10^{-9}$ M, Somatostatin $10^{-5}$ M, Somatostatin $10^{-6}$ M, Somatostatin $10^{-7}$ M, Iodoacetate $10^{-3}$ M, Iodoacetate $10^{-4}$ M, Dinitrophenol $2 \times 10^{-4}$ M, Sodium Chloride $10^{-3}$ M, Sodium Fluoride $10^{-3}$ M, Sodium Azide $10^{-3}$ M, Sodium Cyanide $10^{-3}$ M.

After 60 minutes in the test solution the lung preparation was transferred again to a fresh bath of Krebs-Henseleit saline to find out whether the lung would recover from the treatment. This procedure constituted an ABA experimental design. The control periods were the hour before and the hour after treatment.

In addition, external controls were tested, since in studies of metabolic inhibitors, recovery might be slow or absent. In the external controls, the lungs were transferred in the same manner, but received saline alone in the middle period (Figure 3).

**STATISTICAL METHODS**

Fluid production rates were calculated from the fall in concentration of Blue Dextran. They were estimated from plots of the total volume of fluid against time, with readings recorded every ten minutes; the total volume of fluid was the sum of that within the lungs and that removed for study. The rates of production of fluid over one hour intervals were calculated from the volume plots, using the slopes of their linear regressions, fitted by the method of least squares (Steel and Torrie 1970, in Cassin & Perks 1982; Apple II computer). The significance of changes in rate were estimated from changes in slope, analysed by a t-test for the difference between two regressions (Steel & Torrie 1970; for details see Cassin & Perks 1982). Significance was accepted at $p < 0.05$. Where results from different fetuses were combined, the volumes for each fetus were
Figure 3. Total Volume of Lung Secretion of Untreated Preparations.

Abscissa: total volume of lung secretion, expressed as a percentage of that present at the end of the first hour. The slopes represent the secretion rates; the values below the lines give the average rates in ml/Kg per h of 24 fetuses. The points are graphed with their associated standard errors.
TOTAL VOLUME of LUNG SECRETION (%).

TIME in HOURS.

100% = 0.94 ± 0.06 ml.
expressed as a percentage of the volume at the start of the treatment; the corresponding values were averaged. All mean values are given with their standard errors.

Na\(^+\) and K\(^+\) ions were measured by flame photometer (Instrumentation Laboratories, Inc. Model 343), and Cl\(^-\) ions by a Buchler-Cotlove Chloridometer. The rates of secretion of ions were calculated by the same methods used for secretion of fluid, where total milliequivalents replaced total volume.
RESULTS

Rate of secretion of fluid by fetal guinea pig lungs \textit{in vitro}

Untreated Preparations

The control groups consisted of six or twelve guinea pigs. The secretion rates of each group were averaged for each of the three experimental hours to obtain combined data.

All the control experiments showed a steady increase in the volume of lung fluid throughout the experiments; there were no significant differences in the rates in the different hours. One combined group is shown in Figure 3; the average secretion rates were $1.71 \pm 0.32$ ml/Kg per h for the first hour, $1.54 \pm 0.27$ ml/Kg per h for the second hour and $1.56 \pm 0.26$ ml/Kg per h for the third hour, and there were no significant changes between any of the periods. The results show that the \textit{in vitro} lung continues to secrete for at least 3 hours, with little change, and the rates are comparable to those found in \textit{in vivo} preparations of sheep and goats (see Discussion).

Experimental Groups

a). Sodium iodoacetate

Iodoacetate is a well-known inhibitor of the glycolytic pathway, via the inhibition of fructose diphosphate aldolase (Lehninger 1975).

\textit{Sodium iodoacetate at 10^{-3}M}

All six individual experiments showed a similar reduction in secretion after iodoacetate. In all cases, the reduction was seen in the first hour of treatment, where the change was significant in 4 preparations ($p<0.05-0.001$); in 3 experiments secretion changed to reabsorption. In the final hour all reductions but one were significant ($p<0.05-0.01$), and reabsorption occurred in two preparations; clearly the effect of iodoacetate
persisted after its removal from the outer saline. The results are combined in Figure 4. The figure suggests that iodoacetate abolishes secretion by the isolated lung, with an average depression from $2.65 \pm 0.54$ ml/Kg per h to $0.34 \pm 0.29$ ml/Kg per h in the hour of the treatment and a slight reabsorption of $-0.002 \pm 0.015$ ml/Kg per h in the final hour. Both these reductions were significant at $p<0.001$.

**Sodium iodoacetate at $10^{-4}$M**

Five out of six experiments showed a similar reduction in secretion at a lower concentration of iodoacetate. The reduction was seen in the first hour of treatment, where the change was significant in two preparations ($p<0.05-0.001$); in one preparation secretion changed to reabsorption; however, in one experiment the secretion rate increased significantly. In the final hour all secretion rates decreased, but only two reductions were significant ($p<0.02$). The effect of iodoacetate continued after its removal from the outer saline. The combined results are presented in Figure 4. The figure suggests that iodoacetate reduces secretion by the isolated lung, with an average depression from $2.31 \pm 0.44$ ml/Kg per h to $1.03 \pm 0.57$ ml/Kg per h in the hour of treatment and no recovery in the final hour, when the secretion rate was $0.81 \pm 0.32$ ml/Kg per h. Both reductions were significant ($p<0.05-0.001$).

A rough estimate from the results, by use of their log dose/response relationship, suggests a threshold level at $4.1 \times 10^{-6}$M iodoacetate. The results suggest that glycolysis is necessary for the production of lung fluid in the isolated preparation.

b). **Sodium Fluoride**

These results were checked by the use of an alternative inhibitor of glycolysis, sodium fluoride, which inhibits a different enzyme, enolase (Baldwin 1952, Kessler 1966).
Figure 4. The effect of different concentrations of sodium iodoacetate on lung fluid secretion in fetal guinea pigs.

Abscissa: the total volume of lung secretion, expressed as a percentage of that present at the onset of the treatment, where 100% was:

(a) Na iodoacetate at $10^{-4}$M, 0.8678 ± 0.1689 ml; (b) Na iodoacetate at $10^{-3}$M, 0.8865 ± 0.1109 ml; (c) control, 0.08826 ± 0.0866 ml. Ordinate: time in hours. All regressions are lines of best fit (method of least squares; Steel and Torrie 1970, in Cassin & Perks 1982). The slopes represent the secretion rates; the values below the lines give the average rates in ml/Kg body weight per hour. Asterisks above the line show significant changes (p=0.05 or below) from the original slope (dotted lines). All graphs are averages based on six fetuses.
(a) $n = 6$

2.31 ± 0.44

(b) $n = 6$

2.65 ± 0.54

(c) $n = 6$

2.23 ± 0.43

$10^3$ M Na IODOACETATE

$10^4$ M Na IODOACETATE

Controls

TIME in HOURS.
**Sodium Fluoride at 10^{-3}M**

All six experiments showed reduction in secretion after sodium fluoride. Two of the six showed reabsorption. The change was significant in 5 preparations \((p<0.02-0.01)\). In the final hour, 4 out of the 6 reductions were significant \((p<0.02-0.001)\); reabsorption continued in one preparation. The effect of sodium fluoride continued after its removal from the outer saline. The results are combined in Figure 5. The figure shows that sodium fluoride abolishes secretion by the isolated lung, with an average depression from \(2.96 \pm 0.53\) ml/Kg per h to \(0.2 \pm 0.35\) ml/Kg per h. After the treatment a slight recovery seems to occur; the secretion rate was \(0.31 \pm 0.31\) ml/Kg per h in the final hour. Both these reductions were significant at \(p<0.001\).

c). **Sodium Cyanide**

The possible importance of the aerobic metabolic pathway was then tested by the use of NaCN, which is known to inhibit the cytochrome system and catalase (Keilin 1936a, Keilin & Hartree 1938, Horecker & Stannard 1948).

**Sodium Cyanide at 10^{-3}M**

The effects of NaCN 10^{-3}M were more variable than those of the glycolytic inhibitors. Four preparations showed small reductions in secretion, two of which were significant \((p<0.001)\). The combined data suggests that CN^- has a small effect on secretion rates, with average values falling from \(3.25 \pm 1.18\) ml/Kg per h to \(2.06 \pm 0.75\) ml/Kg per h and to \(2.28 \pm 0.83\) ml/Kg per h in succeeding hours. Both reductions were significant at \(p<0.01-0.001\). The results suggest the aerobic pathways have some influence on the secretion of lung fluid, but the effect is relatively small.

d). **Sodium Azide**

Na azide was tested as an alternative to NaCN, since it also
Figure 5. The effect of sodium fluoride on lung fluid secretion in fetal guinea pigs.

Abscissa: the total volume of lung secretion, expressed as a percentage of that present at the onset of the treatment, where 100% was: (a) Na fluoride at $10^{-3}$M, $0.9840 \pm 0.1199$ ml; (b) control, $0.8826 \pm 0.0866$ ml. Ordinate: time in hours. All regressions are lines of best fit (method of least squares; Steel and Torrie 1970, in Cassin & Perks 1982). The slopes represent the secretion rates; the values below the line give the average rates in ml/Kg body weight per hour. Asterisks above the line show significant changes (p=0.05 or below) from the original slope (dotted lines). All graphs are averages based on six fetuses.
(a) n = 6

(b) n = 6

TIME in HOURS.

TOTAL VOLUME of LUNG SECRETION (%).

Na Fluoride $10^{-3}$ M

Controls

$0.2 \pm 0.35$

$0.31 \pm 0.31$

$2.96 \pm 0.35$

$1.99 \pm 0.73$

$2.23 \pm 0.43$

$2.18 \pm 0.50$
interferes with the aerobic metabolic pathway by actions on cytochrome oxidase and catalase (Keilin 1933, 1936, Keilin & Hartree 1934, Kessler 1966).

**Sodium Azide at 10^-3M**

The results for Na azide were closely similar to those for NaCN. In five out of six experiments, reduction in secretion was found during the period of treatment, and the effect persisted into the final hour. The effect was significant in two preparations (p<0.01-0.001). One preparation showed a rise in the secretion in the period of treatment, but this was not significant, and it fell in the final hour. The combined data (Figure 6) suggests an effect closely similar to that of NaCN with average values of 2.93 ± 0.64 ml/Kg per h in the first hour, 1.2 ± 0.27 ml/Kg per h in the second hour and 1.30 ± 0.54 ml/Kg per h in the third hour. Both these reductions were significant (p<0.01).

The results confirm that the oxidative pathway has some influence on lung secretion, but it does not appear to be as important as the glycolytic system.

e). **Dinitrophenol**

Dinitrophenol was tested as an alternative inhibitor of oxidative processes, since it causes powerful disruption of the mitochondria which carry the enzymes concerned, and uncouples oxidative phosphorylation (Hainstein 1976, Dall-Larsen et al. 1976).

**Dinitrophenol at 2x10^-4M**

All six experiments showed reduction in the secretion after dinitrophenol. Four of them showed reabsorption, two continued to secrete at a low rate. The change was significant in 4 preparations (p<0.02-0.001). Clearly the effect of dinitrophenol persisted after its removal from the outer saline. The results were combined in Figure 7. The figure suggests
Figure 6. The effect of sodium cyanide and sodium azide on lung fluid secretion in fetal guinea pigs.

Abscissa: the total volume of lung secretion, expressed as a percentage of that present at the onset of the treatment, where 100% was:
(a) Na cyanide at 10^{-3}M, 1.0235 ± 0.1752 ml; (b) sodium azide, 0.9410 ± 0.0726 ml; (c) control, 0.8826 ± 0.0866 ml. Ordinate: time in hours. All regressions are lines of best fit (method of least squares; Steel and Torrie 1970, in Cassin & Perks 1982). The slopes represent the secretion rates; the values below the line give the average rates in ml/Kg body weight per hour. Asterisks above the line show significant changes (p=0.05 or below) from the original slope (dotted lines). Averages for (a) and (b) based on six fetuses, average for (c) based on twelve fetuses.
(a) $n = 6$

$$3.25 \pm 1.18$$

Na Cyanide $10^{-3}$ M

(b) $n = 6$

$$2.93 \pm 0.64$$

Na Azide $10^{-3}$ M

(c) $n = 12$

$$2.11 \pm 0.41$$
Figure 7. The effect of dinitrophenol on lung fluid secretion in fetal guinea pigs.

Abscissa: the total volume of lung secretion, expressed as a percentage of that present at the onset of the treatment, where 100% was:
(a) dinitrophenol at $2 \times 10^{-4}$, $0.7852 \pm 0.0647$ ml; (b) control, $0.8826 \pm 0.0866$ ml. Ordinate: time in hours. All regressions are lines of best fit (method of least squares; Steel and Torrie 1970, in Cassin & Perks 1982). The slopes represent the secretion rates; the values below the line give the average rates in ml/Kg body weight per hour. Asterisks above the line show significant changes ($p=0.05$ or below) from the original slope (dotted lines). All graphs are averages based on six fetuses.
that dinitrophenol abolishes secretion and in fact produces reabsorption in the isolated lung, with an average depression from $1.5 \pm 0.43$ ml/Kg per h to $-0.6 \pm 0.17$ in the hour of treatment and continuing reabsorption to $-0.78 \pm 0.23$ ml/Kg per h in the final hour. Both these reductions were significant at $p<0.001$.

f). Piretanide

Piretanide is a loop diuretic which is a potential inhibitor of coupled $\text{Na}^+/\text{K}^+/\text{Cl}^-$ entry across the basolateral membranes of a number of Cl secreting epithelia (Gatzy 1983).

Piretanide at $10^{-7}$M

Five out of six experiments showed reduction in secretion after piretanide. Two of them showed reabsorption. Only one experiment continued secreting in the first hour of the treatment. The change in secretion was significant in 4 preparations ($p<0.02-0.001$). In the final hour all reductions but one were significant ($p<0.05-0.01$) and reabsorption occurred in two preparations. The effect of piretanide persists after its removal from the outer saline. The results are combined in Figure 8. The figure suggests that piretanide abolishes secretion by the isolated lung, with an average depression from $1.96 \pm 0.24$ ml/Kg per h to $0.32 \pm 0.03$ ml/Kg per h in the hour of the treatment and $0.14 \pm 0.01$ ml/Kg per h in the following hour. Both of these reductions were significant at $p<0.01-0.001$.

Piretanide at $10^{-8}$M

Five out of six of the individual experiments showed a slight reduction in secretion with this lower dose of piretanide. In only one experiment it had no effect on the secretion of the isolated lung. The change in secretion was significant in 4 preparations ($p<0.01-0.001$). In the final hour reductions were significant ($p<0.01-0.001$). Clearly the effect of piretanide persisted after its removal from the outer saline. The results
Figure 8. The effect of different concentrations of piretanide on lung fluid secretion in fetal guinea pigs.

Abscissa: the total volume of lung secretion, expressed as a percentage of that present at the onset of the treatment, where 100% was:
(a) piretanide $10^{-7}$M, $0.8745 \pm 0.0627$ ml; (b) piretanide at $10^{-8}$M, $0.8878 \pm 0.1309$ ml; (c) control, $0.8826 \pm 0.0866$ ml. Ordinate: time in hours. All regressions are lines of best fit (method of least squares; Steel and Torrie 1970, in Cassin & Perks 1982). The slopes represent the secretion rates; the values below the line give the average rates in ml/Kg body weight per hour. Asterisks above the line show significant changes (p=0.05 or below) from the original slope (dotted lines). All graphs are averages based on six fetuses.
are combined in Figure 8. The figure suggests that piretanide reduces secretion in the isolated lung with an average depression from $2.4 \pm 0.78 \text{ ml/Kg per h}$ to $1.18 \pm 0.38 \text{ ml/Kg per h}$ in the hour of treatment and $0.67 \pm 0.21 \text{ ml/Kg per h}$ in the final hour. Both reductions were significant at $p<0.001$.

A rough estimate from the results, by use of their log dose/response relationship, suggests a threshold level at $6.6 \times 10^{-10} \text{ M}$ piretanide. The results suggest that this loop diuretic has the effect of reducing the production of lung liquid in the isolated preparation.

g). Somatostatin

Studies of electrolyte secretion by mammalian gastrointestinal tract and pancreas indicate that somatostatin may inhibit secretory transport, and inhibit Cl-secretion (Carter et al. 1978, Epstein et al. 1984).

Somatostatin at $10^{-5} \text{M}$

All six individual experiments showed a reduction in the secretion rate after somatostatin. In two experiments the secretion changed to reabsorption. In all cases the reduction was seen in the first hour of treatment, when the change was significant in 5 preparations ($p<0.01-0.001$). In the last hour the reductions were also significant ($p<0.01-0.05$) in five preparations, and reabsorption occurred in three preparations; clearly the effect of somatostatin persisted after its removal from the outer saline. The results are combined in Figure 9. The figure suggests that somatostatin abolishes secretion by the isolated lung, with an average depression from $3.72 \pm 0.9 \text{ ml/Kg per h}$ to $1.32 \pm 0.32 \text{ ml/Kg per h}$ in the hour of the treatment and a slight reabsorption of $0.07 \pm 0.02 \text{ ml/Kg per h}$ in the final hour. Both these reductions were significant at $p<0.001$.

Somatostatin at $10^{-6} \text{M}$

Five of the six individual experiments showed a similar reduction in
Figure 9. The effect of different concentrations of somatostatin on lung fluid secretion in fetal guinea pigs.

Abscissa: the total volume of lung secretion, expressed as a percentage of that present at the onset of the treatment, where 100% was:

(a) somatostatin $10^{-5}$M, $1.1675 \pm 0.2200$ ml; (b) somatostatin $10^{-6}$M, $0.8747 \pm 0.2229$ ml; (c) somatostatin at $10^{-7}$M, $1.6107 \pm 0.1545$ ml; (d) control $0.8826 \pm 0.0866$ ml. Ordinate: time in hours. All regressions are lines of best fit (method of least squares; Steel and Torrie 1970, in Cassin & Perks 1982). The slopes represent the secretion rates; the values below the line give the average rates in ml/Kg body weight per hour. Asterisks above the line show significant changes ($p=0.05$ or below) from the original slope (dotted lines). Average for (a) based on three fetuses, averages for (b), (c) and (d) based six fetuses.
the first hour of treatment. Only in one experiment did somatostatin have no effect in the secretion of the lung fluid. In three preparations the change was significant \((p<0.02-0.001)\). In the final hour three reductions were significant at \(p<0.001\), and reabsorption occurred in one preparation. It is clear that the effect of these lower doses of somatostatin persisted after its removal from the outer saline. The results are combined in Figure 9. The figure suggests that somatostatin diminished secretion by the isolated lung, with an average depression from \(3.06 \pm 1.06 \text{ ml/Kg per h}\) to \(1.19 \pm 0.54 \text{ ml/Kg per h}\) in the hour of treatment and to \(0.46 \pm 0.49 \text{ ml/Kg per h}\) in the last hour. Both of these reductions were significant at \(p<0.001\).

**Somatostatin at \(10^{-7}\text{M}\)**

Only one of the three experiments showed a decreasing secretion after the lowest dose of somatostatin. The change was not significant at \(p<0.05\). In the final hour only 1 experiment showed a significant reduction of fluid with a \(p<0.05\). The results are combined in Figure 9. The figure suggests that at this level somatostatin has no significant effect in the isolated lung. The average secretion rates were \(1.73 \pm 1.3 \text{ ml/Kg per h}\) for the first hour, \(1.83 \pm 0.61 \text{ ml/Kg per h}\) for the second hour and \(1.95 \pm 0.48\) for the third hour.

A rough estimate from the results, by use of the log dose/response relationship, suggests a threshold level at \(9 \times 10^{-8}\text{M}\), see Figure 10.

**h). Insulin**

Insulin is known to influence the fetal lungs, but could act in opposite ways, since it maintains immaturity of the lung (Tulchinsky & Ryan 1980), which might promote secretion, but can also stimulate Na⁺ transport, which is involved in reabsorption (Macknight *et al.* 1980, Scott & Goodman 1981, Hammerman 1985, Guntupalli *et al.* 1985, in Epple 1987).
Figure 10. The log-dose/response relationship for somatostatin.

A rough estimate from the results, by the use of log dose/response relationship, suggests a threshold level at $9 \times 10^{-8}$ M.
SOMATOSTATIN THRESHOLD

% FALL

THRESHOLD $9 \times 10^4$ M

DOSE
Insulin at $10^{-6}$M

All six individual experiments showed a similar reduction in secretion after insulin. In all cases, the reduction was seen in the first hour of treatment; the change was significant in 5 preparations ($p<0.05-0.001$). In 2 experiments secretion changed to reabsorption. In the last hour all reductions but one were significant at $p<0.05-0.001$. In the two preparations with the reabsorption effect the isolated lung tended to recupeate in the last hour. In general, however, the effect of insulin persisted after its removal from the outer saline. The results are combined in Figure 11. The figure suggests that insulin reduces secretion by the isolated lung with an average depression from $2.47 \pm 0.68$ ml/Kg per h to $0.33 \pm 0.09$ ml/Kg per h in the hour of treatment and down to $0.28 \pm 0.07$ ml/Kg per h in the last hour. Both of these reductions were significant at $p<0.001$.

Insulin at $10^{-7}$M

Five of six of the individual experiments showed a similar reduction in secretion after this lower dose of insulin. One experiment does not show any effect on the secretion rate in the first hour of treatment. The change was significant in 2 preparations ($p<0.01-0.001$). In the final hour 3 reductions were significant at $p<0.01-0.001$. The effect of insulin persisted after its removal from the outer saline. The results are combined in Figure 10. The figure suggests that at this concentration insulin reduces secretion by the isolated lung, with an average depression from $2.63 \pm 0.84$ ml/Kg per h to $1.31 \pm 0.42$ ml/Kg per h in the hour of the treatment and to $0.79 \pm 0.25$ ml/Kg per h in the last hour. Both of these reductions were significant with a $p<0.001$.

A rough estimate from the results, by use of their log dose/response relationship, suggests a threshold level of $4.48 \times 10^{-9}$M of insulin.
Figure 11. The effect of different concentrations of insulin on lung fluid secretion in fetal guinea pigs.

Abscissa: the total volume of lung secretion, expressed as a percentage of that present at the onset of the treatment, where 100% was:
(a) insulin $10^{-6}$M, $0.8362 \pm 0.1271$ ml; (b) insulin at $10^{-7}$M, $0.9248 \pm 0.0362$ ml; (c) control, $0.8826 \pm 0.0866$ ml. Ordinate: time in hours. All regressions are lines of best fit (method of least squares; Steel and Torrie 1970, in Cassin & Perks 1982). The slopes represent the secretion rates; the values below the line give the average rates in ml/Kg body weight per hour. Asterisks above the line show significant changes (p=0.05 or below) from the original slope (dotted lines). All graphs averages based on six fetuses.
DISCUSSION

1). Secretion rates by the fetal lung

The work presented above shows that the fetal lung will continue to secrete in vitro for 3 hours.

Before these studies, extended observations have been made on the secretion of the fetal lung in vivo. In 1953 Reynolds carried out relatively simple experiments. He delivered fetuses from ewes by Caesarean section, maintained the placental circulation, and prevented breathing by applying a rubber nose-bag over the head of the fetus. Large volumes of fluid accumulated in the nose-bag during the period of experimentation, and it was possible to make rough long-term estimates of secretion rates (Reynolds 1953, Avery 1968).

Setnikar and co-workers (1959) performed experiments on fetuses of goats and guinea pigs during the last third of pregnancy. The heads of the fetuses were kept under Ringer solution, and the trachea was cannulated. Displacement of liquid in the respiratory apparatus could be measured by movements of a meniscus in glass tubing, and this allowed measurements of the rate of secretion. However, the values obtained were variable, due to the effects of siphoning and movements of the preparation (Setnikar et al. 1959).

Later workers such as Kitterman et al. overcame some of these problems by collecting lung fluid in bags surgically inserted into fetal lambs, and measuring the accumulation over 12 hour periods. This method made it difficult to measure short-term changes, and impossible to detect reabsorption. However, Kitterman et al. were able to show a progressive decline in secretion rate in the week prior to birth (Kitterman et al. 1979).

The mechanism of fetal pulmonary fluid production appears to be
dependent on active transport of Cl\textsuperscript{-} across the alveolar epithelium. According to Olver & Strang (1974) Cl\textsuperscript{-} transport establishes an osmotic gradient, resulting in movement of water into the alveolus. Olver & Strang (1974) demonstrated that increasing pulmonary blood flow sufficiently to increase pulmonary lymph flow had no effect on lung liquid secretion (Lawson et al. 1978).

Chronically catheterized near-term fetal lambs were used by Cassin et al. (1986b) who infused different substances into the fetal circulation. The secretion rate of the fetal lung liquid was measured by the dye-dilution technique, which had been used by Normand et al. since 1971. With this method it is possible to measure small changes in lung liquid secretion. The technique also allows the detection of reabsorption, and it is convenient for statistical analysis. Average fluid production rates were 3.1 ± 0.3 ml/Kg per h in active experiments in chronic fetal sheep between 128-145 days gestation. However, production rates changed through gestation. Both the 2.2-fold rise to 5.2 ± 0.6 ml/Kg per h at 137-139 days gestation, and the subsequent 83 \% fall by 143-145 days gestation were significant at p<0.01. These results suggest that lung liquid production in chronic fetal sheep falls in the last week before delivery. However, reabsorption over birth is far more dramatic (Perks & Cassin 1985).

In the experiments of Cassin et al. (1986a, 1986b) it was difficult to know the precise concentration affecting the lung; some materials may be rapidly destroyed by the liver and other organs, while some may be lost through the placenta into the large reservoir of the mother (Rudolph 1977). These drawbacks are absent in the in vitro preparation. In addition, the in vitro preparation is an excellent experimental system for testing drugs which may be fatal to the whole animal. The results presented here allow known concentrations to be applied to the in vitro lung, and overcome some
of the problems discussed.

The advantages of using guinea pigs as an experimental animal are that they are small, easy to maintain, they breed all year round, and their gestation period is short (67 days), so we can have rapid results based on large numbers. Their main disadvantage lies in the small volume of fluid which is available for study.

The average secretion rate in the isolated fetal lung during the first hour of all the experiments reported was \(2.31 \pm 0.17\) ml/Kg per h; \(n=104\). This result is close to those reported by the group of Normand et al. (1971) who used the anesthesized fetal lamb (2.5 to 4.0 ml per h), and those of Perks & Cassin (1985b) in chronic fetal sheep (3.1 \(\pm\) 2.2 (SD) ml/Kg per h). The result suggests that the in vitro preparation secretes at an essentially normal rate.

2). Metabolic aspects of lung fluid secretion

The lung is recognized to be a metabolically active and hormonally responsive organ (Strubbs & Alberti 1980, in Kazuhiro et al. 1987). Efforts have been made to characterize the metabolic activities of the lung and to determine how these activities are regulated. Most studies on pulmonary metabolism have used whole lung and focused on glucose and lipid metabolism, which are important for the synthesis of pulmonary surfactant (Van Golde 1976, in Kazuhiro et al. 1987). Amino acids are of obvious importance for protein synthesis and gluconeogenesis (Ballard et al. 1969, in Kazuhiro et al. 1987). In addition to intracellular proteins, Type II cells secrete protein components of the basement membrane and the apoproteins of surface-active material. In Type II cells, amino acids can also be used for lipogenesis, for synthesis of the lipid components of surfactant. Amino acid uptake in the lungs has been investigated in lung slices (Thet et al. 1977, in Kazuhiro et al. 1987), in the isolated perfused lung (Besterman et al. 1987).
1983, in Kazuhiro et al. 1987), and most recently in alveolar Type II cells in tissue culture (Brown et al. 1985, in Kazuhiro et al. 1987). Recently Kazuhiro demonstrated that isolated alveolar type II cells have high affinity insulin receptors and that insulin stimulated glucose transport at physiologic concentrations (Sugahara et al. 1984, in Kazuhiro et al. 1987). Presumably the glucose would be used in metabolic activity, and provide the energy for secretion. Therefore several metabolic inhibitors were investigated, mainly to demonstrate that the fluid movements into the lungs in the *in vitro* preparation depended on metabolic energy, and were not accidental osmotic shifts.

a). *Effects of Sodium iodoacetate and Sodium fluoride*

Sodium iodoacetate was expected to decrease the secretion rate because this drug abolishes the activity of the fructose diphosphate aldolase dehydrogenase and therefore stops glycolysis. Since glycolysis is one of the main sources of energy in the cell it was important to show that the secretion of lung fluid was dependent on this process.

Sodium iodoacetate (10\(^{-3}\)M and 10\(^{-4}\)M) decreased the fetal lung fluid secretion in the guinea pig. A greater depression of the secretion rate was observed at 10\(^{-3}\)M sodium iodoacetate and no recovery took place in the last hour of the treatment. At 10\(^{-4}\)M the secretion rate was also depressed, and no apparent recovery was seen in the final hour.

This result suggests that at least part of the secretion rate of the lung liquid is dependent on glycolysis, since sodium iodoacetate is a potent inhibitor of the secretion of fetal lung fluid at both dose levels.

Sodium fluoride also had a large effect on the production of lung fluid; the secretion was dramatically depressed. The effect was probably due to its known ability to inhibit enolase (Baldwin 1952), and therefore stop glycolysis. Although it also has a small inhibitory effect on catalase, by
forming a well-defined metahemoglobin compound with it, this is probably not its major effect (Keilin 1936).

b). Effects of Cyanide and Azide

For many years it has been known that CN⁻ acts as a powerful respiratory poison, and it was considered to inhibit by combining with the oxidised "Atmungsferment", or "respiratory enzyme" (Keilin 1936). It is now clear that cyanide may inhibit a number of carriers or associated enzymes in the respiratory chain, mainly by forming stable complexes with their Fe-porphyrin compounds (Stannard 1939, in Potter & Bohlender 1941, Commoner 1939, 1940, in Winzler 1943, Lehninger 1979). Although cytochrome b appeared relatively insensitive to cyanide (Keilin 1936, Stolz et al. 1938), small amounts of cyanide (10⁻⁴M) could cause cytochrome a and c to remain completely reduced, and it is now clear that electron transport from cytochrome aa₃ to oxygen is blocked (Dixon 1929, Lehninger 1979). In addition, cyanide can inhibit catalase, the enzyme responsible for the removal of H₂O₂ (Dixon 1929) generated by the respiratory chain, and it also inhibits the indophenol oxidase system (Keilin 1936). In contrast, cyanide does not inhibit aerobic dehydrogenase or systems which react directly with oxygen other than the cytochrome chain (Dixon 1929).

The early claim that the "Atmungsferment" accounted for all respiration of the cell was not born out by later studies, since HCN did not always abolish respiration. In contrast to yeast or Chorella, a considerable number of animal tissues were only inhibited 50-80% by cyanide (Emerson 1927, Dixon & Elliot 1929, in Dixon 1929). An example is the mammalian kidney, where cyanide failed to abolish the capacity for urine dilution or concentration, although it had a natriuretic action due to interference with oxidative processes concerned in sodium transport (Matinez-Maldonado 1969). In many tissues it appears that the cytochrome system does not contribute
more than two-thirds of the total respiration, with the remaining third due to glycolysis, or perhaps aerobic dehydrases (Dixon 1929). The maximum inhibition was usually produced by $10^{-3}$ M HCN, and increasing the concentration 100-fold produced no greater inhibition. The experiments presented here also show that cyanide produced a clear, but only partial inhibition of secretion, which might depend, in part, on oxidative processes. However, before leaving the effects of cyanide, it must not be forgotten that it can poison many systems besides the "respiratory enzyme", for instance peroxidase, catecholoxidase, catalase. Some of the inhibition due to cyanide could be due to these other effects (Dixon 1929).

In 1933, it was shown that sodium azide, $\text{NaN}_3$, inhibits cellular respiration, the oxidation of "cytochrome", and the indophenol reaction in much the same manner as cyanide. However, it was less stable than cyanide, and its effect on the cytochrome system was only marked at a lower pH.

Differences in the cyanide and azide inhibition of tissue respiration have been noted by Stannard (1939 in Winzler 1943) and by Korr (1941 in Winzler 1943), and led them to postulate the existence of by-pass respiration pathways around the cytochrome system. In these early studies, they suggested the existence of different pathways of respiration in resting and stimulated tissues, with part of the resting respiration insensitive to azide. Armstrong and Fisher (1940) have shown that cyanide and azide behave differently in inhibiting the enzymes controlling the frequency of the embryonic fish heart. Ball (1942 in Winzler 1943) has suggested that the Atmungsferment-azide and the Atmungsferment-cyanide compounds might have different oxidation-reduction potentials which could give them different catalytic powers. He pointed out, however, as has also Stolz (1942a in Winzler 1943), that more fundamental knowledge of the mechanism of action of the respiratory inhibitors was necessary before any final interpretations of
their effects could be drawn (Winzler 1943). In addition, it should be remembered that both cyanide and azide also affect the catalase enzyme responsible for peroxide removal along the respiratory chain. However, once again, the mechanisms of the two agents are different (Keilin & Hartree 1934, Keilin & Hartree 1936, Keilin 1936).

In the isolated fetal lung preparation cyanide and azide had a depressing effect on the secretion of the lung fluid. The effect was bigger with azide, but even in this case the lung was still secreting until the end of the experiment. However, it would appear that part of the normal secretion seen in vitro utilized the oxidative pathways.

c). Effect of Dinitrophenol

The first agent found to uncouple oxidative phosphorylation, described by Loomis and Lipman in 1948, was dinitrophenol. Today many different uncoupling agents are known. Most are lipidsoluble substances containing an acidic group and usually an aromatic ring. These agents do not uncouple glycolytic phosphorylation or directly affect cellular reactions other than oxidative phosphorylation. The uncoupling agents allow electron transport to continue, but prevent the phosphorylation of ADP to ATP. Uncoupling agents can promote the passage of H+ ions through the mitochondrial membrane, which is normally impermeable to them (Lehninger 1975).

DNP is an uncoupler of respiration from oxidative phosphorylation both in vivo and in vitro. In early studies, Kotelnikova and co-workers administered DNP to rats and observed a decrease in both ATP levels (Kotelnikova et al. 1960, in Fujimoto, et al. 1964) and turnover (Kotelnikova & Solomatina 1957, in Fujimoto et al. 1964) in liver tissue (Fujimoto et al. 1964). Webster in 1953 (in Grenville & Needham 1955) noticed that DNP in high concentrations, increased the ATPase activity of
myosin. Therefore he began to investigate this effect in the hope that light might be thrown, ultimately, on the interaction of DNP with an enzyme system (Grenville & Needham 1955). In 1976 Dall-Larsen et al. observed that dinitrophenol inhibits ATP phosphoribosyltransferase, seemingly in competition with ATP. Nitrated phenols and other compounds which share the ability to uncouple oxidative phosphorylation have been shown to inhibit several enzymes with adenine-containing substrates or coenzymes (Stockdale et al. 1975, in Dall-Larsen et al. 1976). Previous work has demonstrated that DNP acts as a competitive inhibitor versus ATP in the ATP phosphoribosyltransferase reaction (Dall-Larsen et al. 1975, in Dall-Larsen et al. 1976).

Since oxidative phosphorylation could be important for secretory processes, DNP could be an inhibitor of the transport systems involved. In fact, Dinitrophenol, inhibits sodium transport across a variety of biological membranes (Bricker & Klahr 1966, Handler et al. 1966, Huf et al. 1957, in Martinez-Maldonado 1970). DNP also impairs the renal secretion of organic acids such as PAH, phenol red, and diodrast, in the dog (Mudge & Taggart 1950, Stinckler & Kessler 1963, in Martinez-Maldonado 1970). By contrast, in this species, the excretion of sodium does not seem to be affected when the inhibitor is infused intravenously (Mudge & Taggart 1950, in Martinez-Maldonado 1970), or into a renal artery (Fujimoto et al. 1964, Kessler et al. 1968, Strickler & Kessler 1963, in Martinez-Maldonado 1970). The lack of a DNP effect on sodium reabsorption in the dog has lead to the proposal that a portion of the energy required for this process is not derived from adenosine triphosphate, but that it may come directly from an electron transport system (Kessler 1966). However, in other species the situation appears to be different. Investigators in four different laboratories have shown inhibition of sodium and water reabsorption when DNP
is added to the luminal side of the proximal nephron of *Necturus* (Schatzmann et al. 1958, in Martinez-Maldonado 1970) and of the rat (Chertok et al. 1966, Gertz 1963, Hernandez et al. 1969, in Martinez-Maldonado 1970). However in one situation (frog muscle) DNP has been reported to actively stimulate sodium transport (Conway 1960, in Chertok et al. 1966).

In mitochondria, which carry the oxidative metabolic system, DNP has powerful and widespread effects. In the presence of uncouplers, mitochondrial ATP synthesis is replaced by ATPase activity, respiratory control is abolished, and the free energy of substrate oxidation is dissipated in the form of heat (Poe et al. 1967, in Hainstein 1976). DNP can have other effects: it interacts with several enzymes that handle nucleotides, it inhibits various kinases and dehydrogenases (Stockdale & Selvin 1971, in Harris et al. 1981), it stimulates the myosin ATPase, (Grenville & Needham 1955), and in the case of ATP phosphoribosyltransferase, it can replace ATP as a 'parasite' substrate (Dall-Larsen et al. 1976). The mitochondrial ATPase F₁, is also stimulated by dinitrophenol (Pullmann et al. 1960, Cantley & Hammes 1973, Senior & Tomtsko 1975, in Harris et al. 1981) in an interaction unrelated to its uncoupling activity (Hainstein 1976, Harris et al. 1981).

In the *in vitro* preparation DNP not only reduced fetal lung fluid secretion, as seen in many tissues, but also produced a dramatic reabsorption during the hour of the treatment and in the last hour of the experiment. The persistence of reabsorption after DNP is an unusual observation. However, evidence from adrenaline experiments suggests that reabsorption is dependent on Na⁺ transport. Therefore it seems possible that the fetal lungs resemble the dog kidney, in which DNP did not stop Na⁺ transport, although it inhibited other processes.
d). Effects of Piretanide

Chloride transport is the dominant process for epithelial transport in many tissues, including the loop of Henle, cornea, gastric mucosa, marine telost gill and anterior intestine (Zeuthen et al. 1978). Apparently the lung fluid secretion is also produced by a chloride transport system in the Type II cells (Olver & Strang 1977).

Many of the pumps, channels, and carriers in a cell are amenable to blockade by drugs with varying degrees of specificity and potency. The Na\(^{+}\)-K\(^{+}\)-2Cl\(^{-}\) cotransporter can be inhibited by furosemide, bumetanide, and piretanide. These drugs are collectively referred to as "loop diuretics", because in the kidney they act on the ascending limb of the loop of Henle (Mastella & Quinon 1987). Inhibition by these drugs is rapid and reversible. They are thought to bind to the cotransport protein at the site normally occupied by Cl\(^{-}\). Furosemide is the most extensively studied of the three but bumetanide is now the preferred inhibitor, as it is the more specific and has a potency 50-100 times greater than furosemide (Mastella & Quinon 1987).

Piretanide is an effective inhibitor of electrogenic chloride transport in fish anterior intestine (Zeuthen et al. 1978). Gerencer (1984) obtained similar results in the intestine of a mollusc, and these agreed with studies of the gallbladder by Zeuthen et al. (1978). Although Cassin et al. (1986) showed that bumetanide, and to a lesser extent furosemide, inhibited lung fluid secretion in fetal sheep, they did not test piretanide. This needed to be investigated.

In the in vitro fetal lung, piretanide had a large effect on the secretion of lung fluid. In the two different concentrations that were tested, piretanide slowed the secretion rate in the lower dose and almost stopped it at the higher dose level. This was the first time that piretanide has been tried on the fetal lung. From the results obtained here it seems
that piretanide had a stronger effect than bumetanide and furosemide. We had a reduction in lung fluid secretion at $10^{-8}$M and a bigger effect at $10^{-7}$M. Cassin et al. (1986) found that bumetanide produced reabsorption in the sheep at $10^{-4}$M, and at $10^{-5}$M it reduced secretion; however at $10^{-6}$M there was no clear effect. Furosemide was less effective; at $10^{-3}$M it produced an immediate reabsorption, but at $10^{-4}$M it increased secretion slightly. The use of "loop diuretics" may be particularly important in the treatment of premature babies which suffer from Respiratory Distress Syndrome or Hyaline Membrane Disease, since piretanide and other similar compounds are thought to affect chloride transport and therefore lung fluid secretion.

3) Effects of hormones and related substances on lung fluid secretion

Previous work in the sheep and goat has shown that two hormones, adrenaline and AVP, are capable of affecting lung liquid secretion in the intact fetus in utero. These two hormones also affect the in vitro guinea pig lungs (Marshall, Woods unpublished observations: see Perks & Cassin, 1987). In general, the in vitro preparation presents a useful and rapid method for screening many other hormones, or hormone related substances. Therefore, in this study, two substances not previously investigated, somatostatin and insulin were tested on the in vitro fetal lung.

a) Effect of Somatostatin

There are well over a dozen hormonal substances that are known or strongly suspected to be synthesized in normal lungs. These may have important roles in health and disease. Many of these substances are polypeptides. The site of production of most of these agents is now known (Becker & Gazdar 1984), but their physiological importance is not understood.

Somatostatin is a tetradecapeptide originally isolated from ovine
and porcine hypothalamus. It inhibits the secretion of growth hormone, TSH and prolactin from the pituitary, and can reduce secretion of insulin and glucagon by a direct effect on the islets of Langerhans (Robberecht et al. 1975, Wahren & Feling 1976). In addition, somatostatin is found in the nerves and endocrine cells of the gastrointestinal tract, the endocrine cells of the pancreas, and the parafollicular (C) cells of the thyroid gland (Becker & Gazdar 1984). The presence of somatostatin within the nervous tissue has led to the presumption that it has a peptidergic function (Becker & Gazdar 1984). Somatostatin has been localized throughout the gastrointestinal tract and pancreas, where it exerts a variety of effects on gastrointestinal function. This peptide has been reported to: a) inhibit amino acid and pepsin secretion and delay gastric emptying; b) inhibit the secretion of pancreatic enzymes and bicarbonate; c) diminish gallbladder contractions; d) decrease splanchnic blood flow; e) reduce intestinal motility; and f) inhibit and/or delay glucose and amino acid absorption in humans. In addition, somatostatin inhibits the release of and/or blocks the effect of many hormones which affect fluid and electrolyte transport in the intestine, e.g. VIP, gastrin, secretin, cholecystokinin and GIIP. Therefore, it may function as a neurotransmitter or have a paracrine effect in the intestine. In addition somatostatin functions as a circulating hormone (Becker & Gazdar 1984). Somatostatin stimulates net Na⁺ and Cl⁻ absorption in the rabbit ileum and appears to do so primarily by stimulating the coupled influx of Na⁺ and Cl⁻ across the brush border membrane. Somatostatin was able to block the effect of cyclic AMP-dependent and cyclic AMP-independent secretagogues in the rat colon without affecting cyclic AMP levels, suggesting that it acts by inhibiting a distal step in the secretory pathway, probably involving a final common pathway (Dobbins et al. 1981). Somatostatin may also affect water and electrolyte movement by blocking the
action of cyclic AMP in the rat jejunum (Dharmsathaphorn et al. 1980). Carter et al. (1978) found that somatostatin completely blocked VIP-induced inhibition of water absorption and partially blocked theophylline-induced inhibition of water absorption (in vitro everted sac preparation, rat colon). These studies suggest that somatostatin may modulate fluid movement and the absorption of nutrients from the intestine (Dharmsathaphorn et al. 1980).

Although it has been reported that somatostatin is usually undetectable by radioimmunoassay in normal lungs, it has been found in lung cancer tissue SCCL, and in adenocarcinoma of the lung (Sorenson et al. 1981, Wood et al. 1981, in Becker & Gazdar 1984). However, no studies have been made on fetal lungs, which may retain characteristics of the gut from which the lung developed, and therefore might retain higher levels of somatostatin. In addition, cancer cells, which may represent a less differentiated, and possibly "fetal" condition, are known to synthesize somatostatin in the lung (Becker & Gazdar 1984).

In the isolated fetal lung preparation somatostatin had an effect on the lung secretion rate. The secretion of the lung fluid was stopped at the highest dose level, while the two lower doses reduced secretion. Apparently somatostatin can affect the secretion of Cl- and therefore the secretion of the lung fluid.

b). Effect of Insulin

An increased incidence of RDS occurs in infants born to diabetic mothers (Mestyan et al. 1975, in Giannopoulos & Tulchisky 1980), presumably owing to deficiency of pulmonary surfactant at the time of birth. This finding has stimulated interest in the role of insulin in lung development. Smith and Lumbers (1987) have shown cortisol stimulates the incorporation of choline into lecithin in monolayer cultures of mixed fetal lung cells, and
that this stimulatory effect of cortisol is abolished if insulin is added to the culture medium (Orci et al. 1975, in Giannopoulos & Tulchisky 1980). This suggests that insulin antagonizes the glucocorticoid-induced stimulation of pulmonary lecithin synthesis in the fetus. In other studies (Grasso et al. 1973, in Giannopoulos & Tulchisky 1980), insulin has been shown to stimulate glycogen accumulation and to decrease the number of lamellar bodies in explants derived from 19 old day fetal rat lung in short-term culture, indicating that insulin may delay maturation of the fetal lung. The mechanism involved in such a process is not known, but it has been suggested that insulin may inhibit glycogenolysis and deprive the lung of substrate for phospholipid synthesis. Alternatively, insulin may act by stimulating glycogen synthesis from glucose and thereby again divert substrate away from the production of phospholipids (Grajwer et al. 1977, in Giannopoulos & Tulchisky 1980).

Insulin may play an important role in regulating the metabolism of Type II cells. Clearly, it will be of great importance to assess the interactions between insulin and amino acid transport by these cells in abnormal physiological states, especially starvation or diabetes mellitus. These two states have been reported to affect pulmonary surfactant (Gacad & Massaro 1972, in Kazuhiro 1987), the apoproteins of surface active material (Sugahara et al. 1983, in Kazuhiro 1987), and the ultrastructure of the alveolar type II cell (Sugahara et al. 1981, in Kazuhiro 1987).

The perinatal mortality rate for infants of diabetic mothers has been found to be 6.2% (Lemons et al. 1981, in Smith & Lumbers 1987), an incidence which is greater than in the nondiabetic population. There is also an increased incidence of unexplained fetal death, especially in the last 4 weeks of gestation (White 1974, in Smith & Lumbers 1987), the etiology of which remains unknown. The fetus of a diabetic woman can be exposed to wide
fluctuations in blood glucose levels. Thus, one reason for this unexplained fetal death might be the maternal nocturnal hypoglycemia observed by Guillmer et al. (1975, in Smith & Lumbers 1987). Another explanation could be exposure of the fetus to hyperglycemia. In the normal ovine pregnancy, maternal hyperglycemia caused a rise in fetal plasma glucose levels and increased fetal plasma lactate (Sherlley 1973, in Smith & Lumbers 1987).

In addition to its effect on glucose metabolism and on the fetal lung, insulin can also affect salt metabolism. Herrera in 1963 was the first to observe that insulin stimulates transepithelial sodium transport by the isolated skin of the frog, *Rana pipens* (Herrera 1963, in Cobb et al. 1981). A similar effect was subsequently described in epithelial tissues of the toad, *Bufo marinus* (Herrera 1965, Crabbé & Francois 1967, in Cobb et al. 1981), notably the urinary bladder, a tissue which has transport characteristics in common with the mammalian distal nephron. Although a number of other epithelial tissues, including the mammalian kidney (De Fronzo et al. 1975, De Fronzo et al. 1976, Nizet et al. 1971, in Cobb et al. 1981), responded to insulin by significantly increasing sodium transport, the basic mechanisms leading to this effect are unknown (Cobb et al. 1981). However, there are three main theories to account for this effect: 1) a stimulation of the sodium pump; 2) an unmasking of previously unavailable sodium pump sites, and, 3) a de novo synthesis of proteins which increase the activity of the sodium pump:

1). Stimulation of the sodium pump

Several laboratories have established that insulin, when applied to the serosal side or injected into anurans before removal of the epithelia, stimulates transport of sodium from the apical mucosa surface to the serosa (blood side) of bladder, skin and intestine. This effect is probably due to direct action on the sodium pump at the basolateral cell membrane (Macknight
The isolated urinary bladder of both the toad and the frog can actively transport sodium from the mucosal to the serosal bathing medium. Hormones such as insulin (Herrera 1965, Wiesmann et al. 1977, in Klahr et al. 1981), aldosterone (Crabbé 1961, in Klahr et al. 1981), and antidiuretic hormone (Leaf 1960, in Klahr et al. 1981), all markedly increase this net transport of sodium. The role of insulin on sodium transport has been demonstrated in several other systems including the amphibian colon and skin, and the isolated perfused mammalian kidney. Studies using stripped amphibian colon and skin have suggested a "diuretic" stimulation of short-circuit current after insulin was added to the bathing medium; this was similar to the toad urinary bladder. This stimulation was related to increased activity of the sodium pump rather than to changes in the mucosal permeability of the epithelial cells to sodium. This interpretation has been supported by subsequent observations made by Siegel and Civan (1976) and by Crabbé (1981). Crabbé, who examined the interactions between ouabain, vasopressin, aldosterone and insulin on short-circuit current across the toad bladder, suggested that aldosterone acts primarily to increase sodium entry into the epithelial cells, and that insulin acts primarily to increase sodium extrusion. The experiments of Siegel and Civan also provided evidence that insulin increases sodium transport, at least in part, by directly stimulating the sodium pump (Klahr et al. 1981).

Since insulin stimulates the sodium pump in amphibian tissues (urinary bladder, colon and skin), and these are considered models for certain parts of the mammalian nephron, a similar action of the hormone on the mammalian kidney could be expected. The stimulation of sodium transport in the nephron and functionally related tissues produced by insulin deserves interest in that the hormone apparently fails to influence renal glucose
metabolism or handling (Crabbé 1981).

It is generally agreed that insulin acts, at least initially, as a direct stimulant of sodium transport from the cells to the serosal medium. Evidence includes the fact that insulin and vasopressin (Wiesmann et al. 1977, in Macknight et al. 1980), and insulin and aldosterone (Cox & Singer 1977, in Macknight et al. 1980) together stimulate sodium transport more than supramaximal concentrations of vasopressin or of aldosterone alone. Insulin also stimulates the electromotive force of the sodium pump, $E_{m}$, without affecting cellular resistance (Siegel & Civan 1976, in Macknight et al. 1980). Extensive studies in other tissues support this mode of action (Clausen & Hensen 1977, Gavrick et al. 1975, Moore 1973, Zieler et al. 1966, in Macknight et al. 1980) and suggest that the hormone increases pump activity by increasing the relative sodium affinity of the pump (Clausen & Hensen 1977, Gavrick et al. 1975, Moore 1973, Hougen et al. 1978, in Macknight et al. 1980) rather than by unmasking pump sites (Erlij & Grinstein 1976, in Macknight et al. 1980). In the toad bladder the effect does not depend on serosal medium glucose (Cox & Singer 1977, Wiesmann et al. 1977, in Macknight et al. 1980) nor does it involve cyclic AMP (Wiesmann et al. 1977, in Macknight et al. 1980). The rapid onset of the stimulatory effect of insulin on sodium transport, together with the failure of protein-synthesis inhibitors to prevent this effect (Cox & Singer 1977, in Macknight et al. 1980), suggests that insulin does not stimulate sodium transport by a mechanism involving protein synthesis. In this scenario insulin produces a rapid increase in transepithelial sodium transport apparently as a result of a direct effect in the sodium pump at the basolateral membrane (Macknight et al. 1980). However, this is not accepted by all workers (see below).

2) Unmasking of sodium pump sites

Evidence for a direct effect of insulin on sodium transport in
muscle has been provided by the studies of Moore (1973, in Klahr et al. 1981). Insulin was shown to increase the rate of sodium efflux from a muscle prelabeled with radioactive sodium, an effect that was blocked by ouabain. The effect could not be explained on the basis of changes in sodium permeability. Grinstein & Erlij (1974, in Klahr et al. 1981) demonstrated subsequently that frog muscles exposed to insulin exhibited increased ouabain binding. These observations suggested that insulin unmasked sodium-pump sites previously unavailable for binding of the ouabain molecule (Klahr et al. 1981).

3). De novo protein synthesis

Evidence for de novo protein synthesis after insulin exposure in toad bladder has been presented independently by Benjkamin & Singer (1974, in Klahr 1981) and Cobb et al. (1981). Cobb et al. began their investigation of the mechanism of insulin regulation of sodium transport in the toad bladder by pursuing the finding of Weismann et al. (1976, 1977, in Cobb et al. 1981) that actinomycin D inhibits the prolonged increase in Na⁺ transport produced by insulin. Cobb et al. (1981) provide evidence for the insulin-induced synthesis of specific proteins in one of the two principal morphologic cell types, the granular (G) cell, of the toad urinary bladder. They gave evidence supporting a relationship between induction of these proteins and the prolonged increase in transport caused by insulin. The effect of insulin on Na⁺ transport begins within 15 minutes, but persists for at least 20 hours (Cobb et al. 1981). The relationship of the reported insulin-induced proteins to each other or to (Na⁺+K⁺)-ATPase is uncertain at this time (Klahr et al. 1981).

c). Relationship of Na⁺ transport to the fetal lung

Evidence produced in sheep studies by Brown et al. (1983), confirmed by Perks & Cassin (1987) suggests that adrenaline caused reabsorption in the
fetal lung, and tests with amiloride suggested that this effect was due in part to Na\(^+\) transport from the lumen to the blood. Since Na\(^+\) transport appears to be involved in reabsorption of lung liquid, the effect of insulin, which is itself able to increase Na\(^+\) transport, is in keeping with our understanding of fetal reabsorption at birth. Based on this information we think that the reduction of lung fluid secretion observed in our experimental results in the presence of insulin is due to the stimulation of sodium transport.

The results discussed above suggest that the production of fetal lung fluid was affected by the use of the different metabolic inhibitors. The transport of Cl\(^-\) was inhibited by the use of piretanide, a potent loop diuretic inhibitor. In addition, somatostatin, a hormonal related substance which is known to affect Cl\(^-\) transport, was also found to reduce lung fluid secretion. Insulin, a normally occurring hormone, apparently had no effect on the Na\(^+\) transport in this system.
BIBLIOGRAPHY


Krebs-Henseleit Saline

Method:

Stock Solutions
1). 69.2g NaCl and 3.5g KCl in 1 liter distilled water
2). 36.8g CaCl₂·H₂O in 1 liter distilled water
3). 15.0g MgSO₄ in 1 liter distilled water
4). 16.0g KH₂PO₄ in 1 liter distilled water

The preparation of saline from the stock solutions.

To prepare one liter of saline, mix:

1. 10 ml MgSO₄ stock solution
2. 100 ml NaCl/KCl stock solution
3. 2.1g NaHCO₃
   2.0g Glucose

Fill the volumetric flask at least half full with distilled water. Make sure the solids have completely dissolved.

4. 10 ml KH₂PO₄ stock solution
5. 10 ml CaCl₂·H₂O stock solution

Fill the flask to 1.0 liter with distilled water, and mix thoroughly. Bubble for at least 20 minutes.