THE ROLE OF THYROID AND STEROID HORMONES IN MATURATION OF THE ADRENALINE-SENSITIVE REABSORPTIVE MECHANISM OF THE FETAL LUNG

BY

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ABSTRACT

Around the time of birth, the lung switches from a secretory- to a liquid absorptive organ to enable the fetus to transit from an intra-uterine to an air-breathing environment.

This study concerns hormonal control of the liquid reabsorptive mechanism in the fetal lung which allows this transition to take place. Thyroidectomy in the fetal sheep at 118 days gestation (term = 147 days) prevented the development of adrenaline- or cyclic AMP-sensitivity which , in euthyroid fetuses, resulted in the capacity to absorb lung liquid from 130 days onwards. Studies in which T_3 and T_4 were infused to thyroidectomized fetal sheep showed that T_3 was required for the normal evolution of the reabsorptive response. However, infusion of this hormone to immature fetuses (110 days) did not advance the gestation at which adrenaline-sensitive absorption is first seen. Co-infusion of T_3 and hydrocortisone showed that these 2 hormones have a powerful synergistic effect on the absorption mechanism. Within a few hours of infusion of these 2 hormones to immature fetuses, a reabsorptive response to adrenaline similar to that normally seen in mature fetuses was observed. This response was fully reversible on withdrawal of T_3 and hydrocortisone infusion, and the hormonal effect was blocked by the protein synthesis inhibitor, cycloheximide. These findings suggest that the normal rise in T_3 and cortisol seen in the fetus in late gestation is responsible for maturation of the liquid absorption mechanism which allows the fetus to make a transition to an independent air-breathing existence. These observations may be of significance in the clinical management of infants born prematurely, who may have had insufficient pre-natal exposure to T_3 and cortisol.

CHAPTER 1

INTRODUCTION.

Throughout fetal life, liquid is secreted into the alveolar spaces and conducting airways of the lung. After birth this liquid is replaced with air to allow the lung to function as a gas-exchange organ. The observation that fetal lungs became over-distended with liquid following pathological obstruction of the conducting airways (Potter & Bohlender, 1941) or experimental tracheal ligation (Jost & Pollicard, 1948) indicated that the lungs are filled from within, rather than as a consequence of aspiration of amniotic liquid. The distending pressure generated by the secretory process maintains the fetal lung at a volume equivalent to functional residual capacity (Olver et al, 1973; Nelson, 1966) and is thought to be an important determinant of normal growth and anatomical maturation of the lung (Alcorn et al, 1977). The liquid secreted into the alveolar spaces passes through the fetal trachea and larynx and is then either swallowed or joins the amniotic liquid pool. In the mature sheep fetus, lung liquid is secreted at a rate of about 3 ml/kg/hr (Olver & Strang, 1974) with similar rates reported for fetal goat (Perks & Cassin, 1985), fetal guinea pig (Perks et al, 1990) and fetal rat alveolar explants (Krochmal et al, 1989).

The chemical composition of fetal lung liquid in fetal sheep and guinea pig is quite distinct from that of amniotic fluid, plasma or the lymphatic fluid of the lung. Its principal characteristics are that it has a high chloride and potassium, and low bicarbonate ionic composition, with a very low protein concentration (Adamson et al, 1973; Perks et al, 1990). When measured with respect to the plasma compartment, the alveolar lumen has a negative electrical potential of about -4 mV (Olver and Strang, 1974).

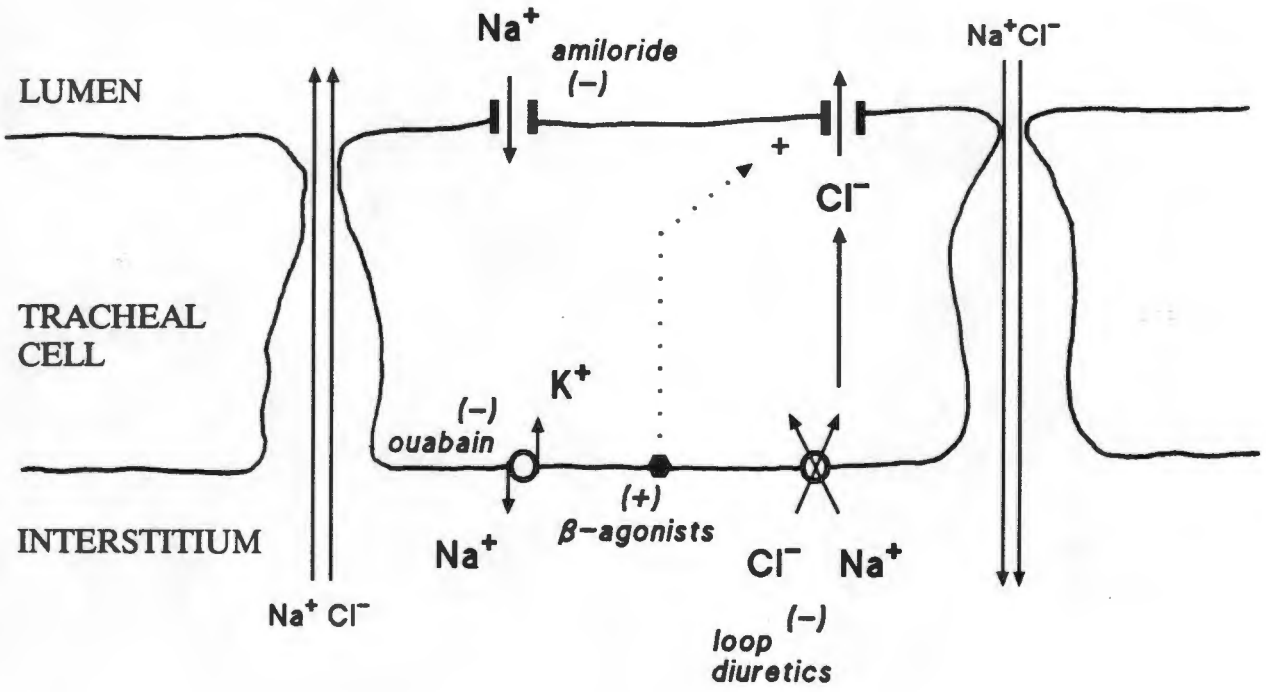
The studies with whole animal models have produced information about the net secretory pattern of the whole lung since it is not possible to determine the relative contribution of various regions of this organ. It is assumed, however, that these studies reflect predominantly ion transport across primitive alveolar saccules in view of the overwhelming contribution of this region, even at the gestations studied, to the total surface area of the lung. The ion transport characteristics of the very distal airways epithelium are not known and may exert a significant influence on overall liquid transport, particularly in very early gestation when the primitive distal pulmonary structures contribute a smaller fraction to overall surface area. The dominant role of the distal lung in ion and liquid transport is further indicated by the net liquid absorptive response to intravenous adrenaline infusion of the mature fetal sheep lung *in vivo* (Brown et al, 1983). This effect occurs in the face of an opposing net Cl^- secretory response to adrenaline which is observed in the isolated fetal trachea of the same species (Olver & Robinson 1986), indicating that the reabsorptive response to these agonists represents events occuring in the distal lung.

Studies on fetal sheep established that lung liquid secretion is associated with active transfer of Cl^- ions from the interstitial to the luminal side of the pulmonary epithelium (Olver & Strang, 1974). Secretion of lung liquid ensues when Na^+ and water follow Cl^- passively to maintain electrochemical neutrality and isosmolarity. Ionic flux studies of the fetal sheep trachea are contradictory with some workers finding evidence for Cl^- secretion in the resting state (Cotton et al 1983) and others reporting that Cl^- secretion was present only during

stimulation with a ß-agonist (Olver et al, 1986). In a later study Cl^- secretion was found to be sensitive to loop diuretics (Cotton et al 1988). The exact mechanism by which ions are transported across the distal pulmonary epithelium of the fetus occurs is not known. Our current model (Olver et al, 1986) is based on a similar model for ion transport across respiratory epithelia of the fetal and postnatal conducting airway (Frizzell et al, 1979, Widdicombe et al, 1979). The electrochemical gradient created by the Na^+/K^+ ATPase pump at the basolateral membrane generates a chemical driving force for Na^+ to re-enter the cell. It is proposed that this ion does so together with Cl^- via a bumetanide-sensitive electroneutral carrier on the basolateral membrane. Cl^- then exits the apical membrane of the cell into the alveolar lumen through putative Cl^--selective channels down its electrochemical gradient. Na^+ and water follow Cl^- across the cell to maintain electrical neutrality and isosmolality, resulting in secretion of lung liquid (fig 1).

There is evidence for the existence of a ouabain-sensitive Na^+/K^+ ATPase mechanism in fetal rat alveolar explants (Krochmal et al, 1989), and evidence for similar ion exchange mechanism in primary isolates of fetal rabbit Type II cells (Bland & Boyd, 1986). There is also some supportive evidence that the secretory process in the fetal sheep lung is mediated by Cl^- transport in that the secretion rate falls in the presence of loop diuretics (Cassin et al, 1986).

Figure 1 Model for transport of Na^+ and Cl^- across the fetal sheep trachea.



Successful transition from a liquid-filled fetal lung to an air-filled, gas-exchanging organ at the time of birth requires the alveolar spaces to be rapidly cleared of liquid and the secretory process to be brought to a halt. In fetal sheep there is a gradual decline in lung liquid volume and slowing of the secretion rate in the last few weeks of gestation (Dickson et al, 1987; Brown et al, 1983). At the start of labour, however, the fetal lung lumen still contains about 20 ml of liquid/kg fetus. The observation that rabbit pups born at term by Caesarian Section before labour had significantly greater lung water content than the pups born following labour either by Caesarian Section or the vaginal route (Bland et al, 1979)

suggests that the process of labour itself influences the amount of liquid present in the lung at birth rather than the route of delivery. The syndrome of Transient Tachypnoea of the Newborn is more prevalent in babies born via elective Caesarian Section and has been attributed to delayed clearance of fetal lung liquid (Avery et al, 1966). It is now thought unlikely that expulsion of liquid via the upper airway due to compression of the thorax during vaginal delivery accounts for significant removal of residual lung liquid.

It has now been established that rapid clearance of the fetal lung can be achieved by re-absorption of liquid from the alveolar spaces across the pulmonary epithelium (Walters & Olver, 1978). This reabsorptive process is initiated during spontaneous labour and continues during delivery and following birth (Brown et al, 1983). In this by study by Brown et al, there was a close correlation between the rates of reabsorption during labour and the concentrations of adrenaline in fetal plasma. As a consequence of the stress of labour and delivery fetal plasma adrenaline levels increase by over an order of magnitude over basal levels. The reabsorptive mechanism can be studied in isolation (outside the context of labour) since reabsorption of lung liquid can be activated experimentally in resting mature fetus sheep by infusion of adrenaline into the fetal circulation (Walters & Olver, 1978).

The capacity of the pulmonary epithelium to reabsorb liquid following a given rise in fetal adrenaline concentration (0.1 to 1.0 ng/ml) increases dramatically during the last 10% of gestation in the fetal sheep (Brown et al 1983). Whereas this experimentally- induced rise in adrenaline concentration results in only a small fall in the resting secretion rate in fetuses

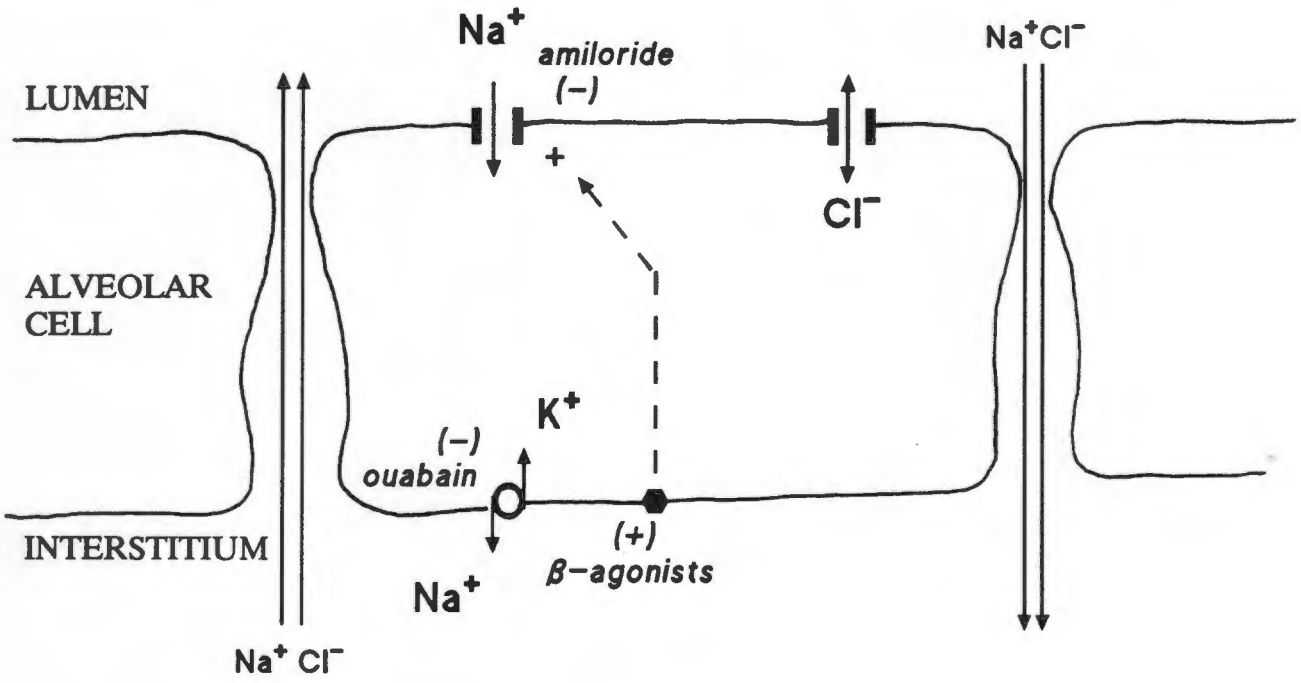
less than 130 days gestation, a similar rise brings the secretory process to a halt around 130 days, and induces reabsorption of increasing magnitude thereafter. After the adrenaline infusion is discontinued, reabsorption of lung liquid ceases and the normal secretory state resumes. The reabsorptive response can also be induced by the addition of dibutyryl cAMP to fetal lung liquid and the magnitude of this response also shows a striking increase with gestation which closely parallels the increasing sensitivity to adrenaline (Olver et al, 1987).

It is now known that ß-adrenergic stimulation induces this reabsorptive effect in the mature fetus by provoking transport of Na^+ ions across the pulmonary epithelium from the lumen to the interstitium (Olver et al, 1986). Na^+ concentration within the cell is kept low by Na^+/K^+ ATPase on the basolateral (interstitium-facing) border of the cell. The channels which open or are activated on the apical (lumen-facing) membrane by adrenaline stimulation allow Na^+ to enter the cell rapidly down its concentration gradient. Na^+ then exits rapidly through the basolateral membrane via the Na^+/K^+ ATPase pump (fig 2). Cl^- and water follow from the lumen to maintain electrochemical and osmotic neutrality resulting in net absorption of lung liquid. Activation of this Na^+ transport can be inhibited by micromolar concentrations of amiloride, a known blocker of Na^+ channels, and is completely reversible following withdrawal of the adrenaline stimulus.

While no agents have been found to stimulate lung liquid production, partial inhibition of secretion has also been achieved with the use of indomethacin (Cassin, 1983), epidermal growth factor (Kennedy et al,1986) and vasopressin (Ross et al, 1984, Wallace et al, 1990).

The physiological role of these factors has not been established.

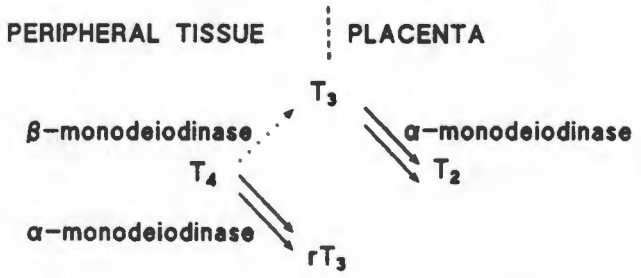
Figure 2. *Proposed mechanism for adrenaline and cAMP activated Na^+ absorption.*



This investigation focuses on two hormones which were considered to be likely candidates to influence maturation of the reabsorptive capacity of the fetal lung in late gestation. In addition to having maturational effects on other aspects of fetal lung development (see below) we noted that the gestation at which the lung liquid reabsorptive response to adrenaline first appears in fetal sheep coincides with increases in the concentrations of both these hormones in fetal plasma.

Thyroid hormones. Only a small proportion the deiodination products of T_4 (the active

hormone T_3 and its inactive counterpart rT_3) are secreted by the fetal thyroid gland - the major fraction of these two hormones being derived from peripheral deiodination (Chopra 1976) of circulating T_4. Throughout most of fetal life in the sheep and in other mammalian species, T_4 is deiodinated predominantly to rT_3 (Chopra et al 1978) so that levels of this inactive hormone are relatively high compared to T_3. This relationship has been attributed to a relative predominance of α-monodeiodinase in the peripheral tissues and placenta over ß-deiodinase (Huang *et al*, 1988, Wu *et al*, 1978). High α-monodeiodinase concentration in the placenta ensures that any T_3 that crosses from the maternal circulation is rapidly converted to T_2. Through these mechanisms the fetus is protected from the effects of T_3 until the latter part of gestation, probably to conserve fetal energy metabolism.



Maturation of the ß-ring deiodination pathway does not take place to any significant extent in the fetal sheep until after 130 days gestation (Wu *et al* 1978) so that fetal plasma concentrations of T_3 remain low (0.2 ng/ml or less) in the sheep until the last three weeks of gestation during which time a gradual rise in T_3 concentration to about 0.5 ng/ml and a corresponding fall in concentration of rT_3 is seen (Fisher et al 1977, Fraser and Liggins 1988).

These changes are thought to result from an increase in ß-ring monodeiodinase activity in peripheral tissues (Wu et al, 1978) and a simultaneous decrease in alpha monodeiodinase activity observed in rat skeletal muscle and intestine (Huang et al, 1988) and sheep placenta (Roti et al, 1982). The high metabolic clearance rate of fetal T_3 reported by Fraser and Liggins (1988) probably reflects the activity of the latter enzyme.

Thyroid hormones, thyroxine (T_4) and triiodothyronine (T_3), have been shown to influence morphological development (Rooney et al, 1975, Korda et al, 1984) of the fetal lung and maturation of the surfactant system (Hitchcock et al, 1979, Nwosu et al, 1980) in a range of mammalian species.

Fetal cortisol levels: Fetal plasma cortisol concentrations are likewise low throughout most of gestation principally as a result of conversion by the placenta of fetal and maternal cortisol to its inactive metabolite cortisone (Buster, 1983). A gradual rise in fetal cortisol concentrations is seen in the fetal sheep from about 130 days (Ballard et al, 1982). Nathanielsz (1972) reported a later, more pronounced rise in cortisol concentrations to 10 ug/dl only in the last few days before delivery.

The maturational influence of corticosteroid hormones on anatomical development of the fetal alveolus and surfactant production has been even more extensively studied than that of thyroid hormones (for review see Ballard, 1986). In addition to their independent effects, thyroid and steroid hormones have been shown to act synergistically to increase the appearance rates of lamellar bodies of fetal type II cells, surfactant secretion into lung liquid

(Hitchcock, 1979; Torday & Dow, 1984; Schellenberg et al, 1988) and improve lung distension of newborn sheep treated with these hormones in-utero (Schellenberg et al, 1988, Warburton, 1989).

Both thyroid and steroid hormones are known to influence ion transport in other epithelia. Thyroid hormones have been shown to exert an indirect effect on both Na^+ and Cl^- transport via a stimulatory effect on the Na+/K+ ATPase pump (for review see De Groot *et al*, 1986). Glucocorticoids have been shown to augment both amiloride blockable Na^+ transport in the collecting duct of rat kidney (Husted et al, 1990) and Na^+/H^+ exchange in renal microvilli, as well as Na^+/Cl^- co-transport in the rat colon (Turnamian et al, 1990). Although an additive effect of thyroxine and aldosterone has been shown on K^+ transport in *Xenopus* lung, there are no reports of a synergistic effect of thyroid hormones and glucocorticoids on ion transport. In addition, none of the above effects of hormones on ion transport systems in other organs could account for the development, in late gestation, of the liquid absorptive response to adrenaline infusion in the fetal sheep lung. This unique ion transport system allows continued net secretion of liquid (via Cl^- secretion) while the epithelium is primed to respond to adrenaline with an increasing net liquid absorptive response (via Na^+ absorption) in the last 3 weeks of gestation.

This study derives from previous studies of the fetal lung in sheep conducted over a 25 year period in the Department of Paediatrics at University College London under the direction of Prof L.B. Strang. The fetal sheep is a particularly good model for studying the

mechanisms of ion and liquid transport in the lung. Its structural and morphological development is well described (Ten Have-Opbroek, 1981) and the gestation at which this study was carried out (120 to 147 days) is equivalent to the mid-second trimester onwards in the human fetus. In addition the sheep fetus at this gestation is large enough to allow cannulation of the trachea and neck vessels for experimental purposes and is hardy enough to withstand operative and experimental procedures without ill effect.

Individual fetuses were studied longitudinally through the latter part of gestation following implantation of catheters into the fetus *in-utero*. This allowed multiple experiments to be conducted on the same fetus following recovery from surgery.

This study concerns possible hormonal influence on the maturation process which results in an increasing adrenaline-sensitive reabsorptive capacity of the fetal pulmonary epithelium during late gestation. My strategy was, firstly, to observe the effect of removal of fetal hormone on maturation of the reabsorptive response, and then to attempt to manipulate the reabsorptive capacity by selectively replacing T_3, T_4, and cortisol - alone or in combination.

CHAPTER 2

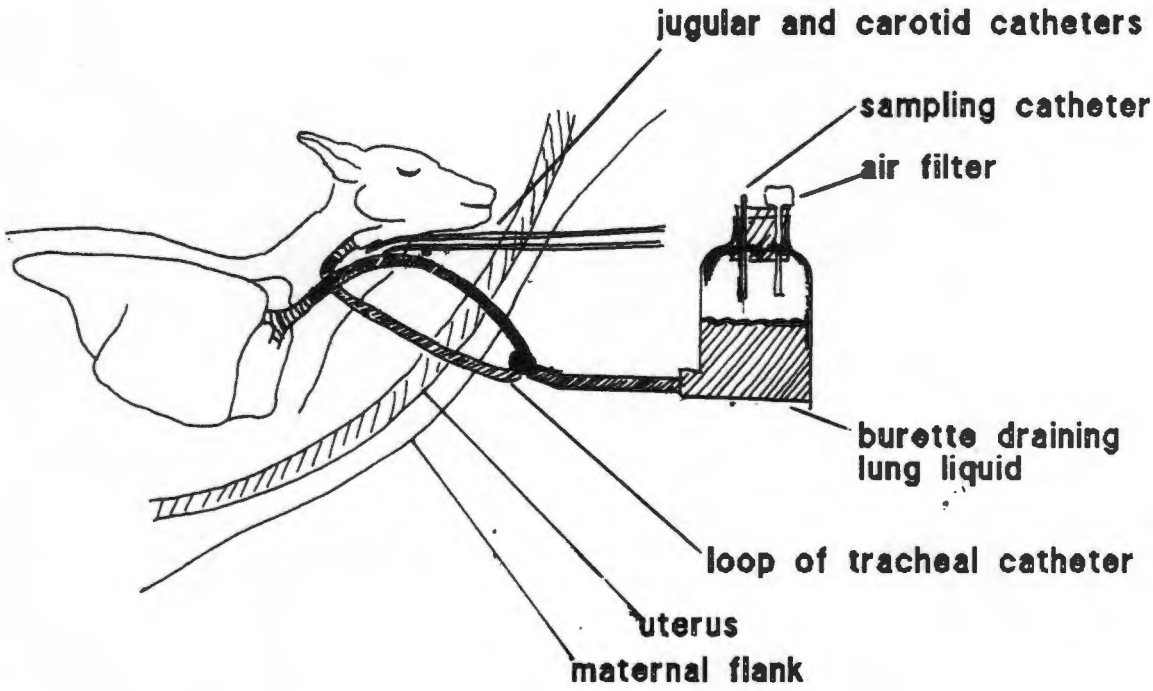
METHODS

Operative procedure: Fetal catheterisation and thyroidectomy.

Pregnant Dorset Short Horn or Clun Forest ewes of known tupping dates from one supplier were used. The basic surgical procedure followed that described by Walters and Olver (1978).

Anaesthesia of the pregnant ewe and her fetus was induced with intravenous Sodium Thiopentone and maintained by inhaled fluothane (1.5 to 2.5%) in a closed circuit. Following a midline abdominal incision the fetal head and neck were delivered through a small hysterotomy incision taking care to minimise amniotic fluid loss. The nose and mouth were covered with warm saline soaked gauze to prevent fetal gasping. Fetal thyroidectomy was performed through a midline neck incision after identification and isolation of both recurrent laryngeal nerves and cautery of the vessels leading to the gland. The fetal trachea was incised between the 3rd and 4th cartilaginous rings leaving the membranous part intact. Into each end of the incised trachea a wide bore silastic catheter (Dow-Corning, internal diameter 0.26 cm) was tied and finer gauge silastic catheters were inserted and tied into the left or right jugular vein and carotid artery. The uterus and abdomen were closed in layers and the catheters were led through a subcutaneous tunnel to emerge through the maternal flank some distance from the abdominal wound.

Figure 1. *Fetal sheep with tracheal, carotid and jugular catheters in-situ. Proximal limb of tracheal loop is clamped to allow lung liquid to drain into burette.*



The large catheters originating in the proximal and distal parts of the incised trachea were joined so that a continuous loop existed which allowed liquid formed in the lung to travel unhindered to the larynx and on to the mouth and amniotic pool. The venous and arterial catheters were filled with heparinised saline (500 units heparin/ml saline). All catheters were then wrapped in swabs soaked in 70% ethanol and stored between experiments in a sterile polythene bag tied to the back of the ewe. Both ewe and fetus received a 2 day post-operative course of Benzyl Penicillin (300 mg/day i.m. to ewe, 70 mg/day i.v. to fetus) and Streptomycin (500 mg/day i.m. to ewe, 120 mg/day i.v. to fetus). No experiments were performed in the 2 day post-operative interval while the ewe and fetus were receiving prophylactic antibiotics.

The purpose of the experiments was to make repeated measurements of lung liquid secretion or absorption rates before and during adrenaline infusion in individual fetuses at different gestations. Experiments were conducted under sterile conditions.

Measurement of lung liquid secretion or absorption rates.

At the start of each experiment the exteriorised loop of catheter containing fetal lung liquid was interrupted with a clamp and the proximal and distal limbs of the catheter loop were isolated. The distal (lung) end of the catheter was connected to a glass burette and about half of the liquid in the fetal lung could be syphoned off by lowering the receptacle below the level of the fetus. At the start of the experiment an impermeant tracer (^{125}I-albumin, 1 - 2 μCi) was added to the collected liquid. By repeated elevation and lowering of the burette (every 2 to 3 minutes) even mixing of this tracer in the liquid throughout the lung was maintained for the duration of the experiment. The adequacy of mixing has been established previously by showing that diffusion of impermeable test substances of different molecular radii (albumin and inulin) in lung liquid was not a factor in determining concentration changes of these substances resulting from secretion or absorption of lung liquid (Normand et al, 1971). The use of a glass burette was a modification of the method described by these authors in which a 50 ml syringe was used to mix the lung liquid.

During the first of 30 minutes of the experiment the tracer was mixed thoroughly into the liquid compartment of the lung. Seven samples (0.5 ml each) of the liquid were taken at 5 minute intervals for the next 30 minutes to determine the resting lung liquid secretion rate.

Adrenaline (0.5 ug/min) was then infused through the fetal jugular catheter; 15 minutes after the commencement of this infusion a further 7 samples were taken at 5 minute intervals to allow calculation of the new secretion or absorption rate. In some experiments dibuteryl cyclic AMP (db-cAMP, 5 mg) was added to lung liquid in place of the adrenaline infusion. This gave a final concentration of db-cAMP in lung liquid after mixing of 10^{-4}M. In these experiments secretion or absorption rate before and 90 to 120 minutes after the addition of db-cAMP was measured. At the end of each experiment sulfamethazine (165 mg) was both added to lung liquid and infused into the fetal circulation to prevent infection. The loop of tracheal catheter was then re-established to allow free passage of lung liquid to the larynx.

Calculation of lung liquid secretion or absorption rates.

Weighed samples containing ^{125}I albumin were counted on a Packard Autogamma Spectrometer to determine the concentration of this tracer in lung liquid. The background radiation and residual radioactivity from previous experiments were subtracted from the ^{125}I counts in the experimental samples. Where V_s is the sample volume, ΣV_s the volume removed in samples up to time **t**, **R** the amount of tracer in these samples, **x** the amount of tracer introduced at $t = 0$, and C_t the concentration of tracer at time **t**, then the cumulative volume of lung liquid at time **t** (V_t) is given by:

$$V_t = \Sigma V_s + (x - R)/C_t$$

Cumulative volume is the sum of the liquid in the lungs at time t plus the liquid volumes

removed in samples up to t. The slope of V_t on t, calculated by least squares, gives the lung liquid secretion rate (J_v) or the absorption rate ($-J_v$).

The secretion rates obtained by previous workers in this laboratory using this method correlate closely with rates obtained by collecting liquid directly over long periods from a cannula chronically implanted in the fetal trachea (Platzker et al, 1975) or by measuring the flow of liquid through the trachea with a bubble flowmeter (Adamson et al, 1975).

Monitoring of the fetus during the experiment.

During the experiment regular measurements of arterial blood gas tension, heart rate and blood pressure were made. The blood pressure transducer was connected to the fetal arterial catheter and fixed at a level which was judged to be approximately the height of the fetal heart. This did not allow comparison of the absolute systolic and diastolic blood pressure but permitted measurement of changes in mean blood pressure before and during adrenaline infusion. In some fetuses there was evidence of partial or complete blockage of the arterial catheter and in these instances the tracings of heart rate and blood pressure were not used for analysis. Arterial pH, pCO_2 and pO_2 were measured directly after sampling in a heparinised syringe (50U heparin/ml blood) using an ABL1 blood gas analyser (Radiometer, Copenhagen)

Hormone infusions into the fetal plasma.

Hormones were delivered as a continuous infusion (Graseby Syringe Driver, type MS16a)

into the fetal jugular catheter and in some instances the infusion of T_3 or hydrocortisone was initiated with a bolus injection of hormone given by hand (see Chapter 6). Adrenaline was diluted to 5μg/ml in normal saline and delivered at 0.5μg/min over 45 minutes (4.5 ml). The total daily dose of T_3 (30, 60 or 120 μg), T_4 (50 μg) or hydrocortisone (10 mg) was diluted to 10 ml in sterile water and delivered over 24 hours. The dead space of the catheter (1.2 ml) was filled with the infusate before infusion was commenced.

Thyroid hormone and adrenaline concentrations in fetal plasma.

T_4 and T_3 concentrations were measured using a commercially available (Ammerlex-M, Ammersham) radioimmunoassay (RIA) kit. The lower limit of detection for T_4 was 3.1 ng/ml and for T_3 was 0.09 ng/ml. Reverse T_3 (rT_3) was likewise measured with a commercial radioimmunoassay kit (Serono); the lower limit of detection was 0.02 ng/ml. Samples from non-thyroidectomised fetuses were diluted 4 times to increase accuracy at the upper limits of detection of this assay (2 ng/ml) as relatively high levels of rT_3 are normally seen in the sheep fetus. The lower limit of detection for the cortisol assay was 0.5 μg/dl. Samples for the radioimmunoassays were measured in duplicate and the meaned value of radioactive counts was read off a standard curve constructed from standards provided for each kit. The average variation about the mean of each paired sample for all 4 hormones was 3.1$\pm$0.7%. The intra-assay coefficient of variation (n = 10 for each test) for the RIAs at the lower and higher ends of the ranges tested were: T_3 3.7% and 2.5%; T_4 3.2% and 3.3%; cortisol 4.9% and 3.7%. Plasma adrenaline levels were measured by a double isotope modification (Brown and Jenner, 1981) of the radioenzymatic method of Da Prada and

Zurcher (1976). Samples of fetal blood taken for hormone estimation during the experiment were centrifuged at 4°C and immediately stored at -70°C until the time of analysis.

Calcium concentrations in plasma and lung liquid.

Samples of arterial fetal blood and lung liquid were taken for determination of calcium concentration. Measurements were made on a Technicon Smac computer-controlled biochemical autoanalyser. These levels were corrected for differences in plasma albumin between Thyroidectomised (Tx) and non-Tx fetuses to a standard albumin concentration of 27 g/l (the mean albumin concentration in the non-Tx fetuses). Lung liquid calcium concentration was measured on a Roche-Cobas-Bio centrifugal analyser.

Drugs used.

T_3 and was obtained from Hennig, Berlin, FRG, and Glaxo, England. T_4 was obtained from Hennig and was determined to contain less than 0.01% T_3. Adrenaline was manufactured by Antigen, Ireland and Hydrocortisone by Upjohn, England.

Statistics.

Unless otherwise stated, all values given are mean ($\pm$ standard error of the mean). Paired and non-paired data were compared using paired and non-paired Student's T tests. Regressions were calculated using the least squares method. Statistically significant results assume a P value of < 0.05.

Control data.

Experiments on non-thyroidectomised fetuses (Brown et al, 1983; Olver et al, 1987) were carried out in our laboratories using an identical experimental protocol to that outlined above provided control data for adrenaline responsiveness in this study. The justification for the use of historical controls is that the studies reported in this thesis lead directly from work done in the same laboratory just prior to my arrival at University College and follow the basic operative and experimental format which are is identical to that previously used on sheep of the same breed and from the same supplier. Control thyroid hormone and calcium concentrations were measured in samples taken from chronically catheterised non-thyroidectomised fetuses undergoing an investigation of glucose transport in the fetal lung (Barker et al, 1989).

CHAPTER 3

THE EFFECT OF THYROIDECTOMY ON THE ADRENALINE-SENSITIVE LUNG LIQUID REABSORPTION MECHANISM.

In order to determine whether endogenous fetal thyroid hormones play a role in development of the reabsorptive response in the fetal lung, we performed thyroidectomy in 15 fetal sheep at between 113 and 120 days gestation (median 118 days) - some 2 weeks before the reabsorptive response to adrenaline is normally observed (Brown *et al*, 1983). This allowed us to compare the maturation of the reabsorptive response to adrenaline and to cyclic AMP in a group of hypothyroid fetal sheep with that taking place in a group of euthyroid fetuses.

Thyroid hormone concentrations

Table 1 gives values of thyroid hormone concentration in thyroidectomised (Tx) and control (non-Tx) fetuses. Control hormone concentrations were measured in samples taken from non-Tx fetuses which had been subjected to the same surgical procedures as the Tx fetuses, except for thyroidectomy, and which were undergoing similar experiments in another project (Barker et al, 1989).

In fourteen out of fifteen of the Tx fetuses, levels of thyroxine (T_4), tri-iodothyronine (T_3) and reverse T_3 (rT_3) were near to or below the lower limits of detection for the respective assays. This was in clear distinction to levels measured in the control group which were in

the range expected for normal sheep fetuses at the gestations studied (Nathanielsz et al, 1973; Fisher et al, 1977; Nwosu et al, 1979; Fraser and Liggins, 1988).

Table 1 Thyroid hormone levels

gestation (days)		T_4 (ng/ml) non-Tx	T_4 (ng/ml) Tx	T_3 (ng/ml) non-Tx	T_3 (ng/ml) Tx	rT_3 (ng/ml) non-Tx	rT_3 (ng/ml) Tx
120-129	mean	98.8	7.2	0.21	0.09	3.20	0.09
	s.e.m.	±10.6	±0.8	±0.03	-	±0.33	±0.03
	n(total)	5	29	10	18	5	16
	n(<det^n)	0	6	0	18	0	0
130+	mean	70.6	5.6	0.36	0.09	3.94	0.04
	s.e.m.	±21.1	±0.7	±0.05	-	±0.29	±0.02
	n(total)	8	27	17	11	5	15
	n(<det^n)	0	5	0	11	0	3
	fetuses	4	14	8	7	5	9

n(s) = total number of samples; n(<d) = number of samples below limits of detection. Lower limits of detection (ng/ml) were: T_4 = 3.10, T_3 = 0.09, rT_3 = 0.02. These levels were used in calculating the means when the concentraton was below the limit. Hence the means for the Tx fetuses are overestimated.

In one thyroidectomized fetus there was biochemical evidence of regeneration of thyroid function in late gestation. In this fetus T_4 levels rose from 5ng/ml at 127 days to 26ng/ml at 145 days. At this gestation mean T_4 levels in the other Tx fetuses were about 5 ng/ml and in the non-Tx fetuses were about 55ng/ml. The results from this fetus are not included in Table 1 and the effects of adrenaline infusion were analysed separately from the others in the thyroidectomised groups.

Calcium concentrations

To determine effects of possible inadvertent parathyroidectomy in the Tx fetuses, we measured total calcium levels in plasma and lung liquid of both Tx and non-Tx fetuses. Plasma albumin (g/l) was significantly lower in the Tx fetuses (mean in Tx fetuses, 22.2 $\pm$0.4, n = 25; mean in non-Tx fetus = 27.2$\pm$0.6, n = 8). When allowance was made for the difference in albumin concentrations, the mean plasma calcium value in the Tx fetuses (2.92$\pm$0.09 mM, n = 25) was not significantly different to that in the control group (3.11$\pm$0.06 mM, n = 8). There were likewise no significant differences in the mean calcium concentrations in lung liquid (Tx = 0.35$\pm$0.02 mM, n = 40; non-Tx = 0.36$\pm$0.03 mM, n = 25).

The effect of adrenaline infusion

Fetal lung liquid secretion rate was measured before and during intravenous infusion of adrenaline (0.5 ug/min) at various gestational ages. Table 2 gives the results of these experiments in Tx fetuses (excluding the fetus showing evidence ofthyroid regeneration) and of experiments on non-Tx fetuses from Brown *et al.(1983)*. At gestations below 130 days, the mean resting secretion rate ($J_v c$) in the Tx fetuses was significantly lower than in non-Tx fetuses but in the mature groups these rates were not significantly different.

Table 2. *Mean (±s.e.m.) secretion rates before ($J_v$control) and during ($J_v$adren) infuson of adrenaline at 0.5ug/min in euthyroid (non-Tx) and thyroidectomised (Tx) fetuses*

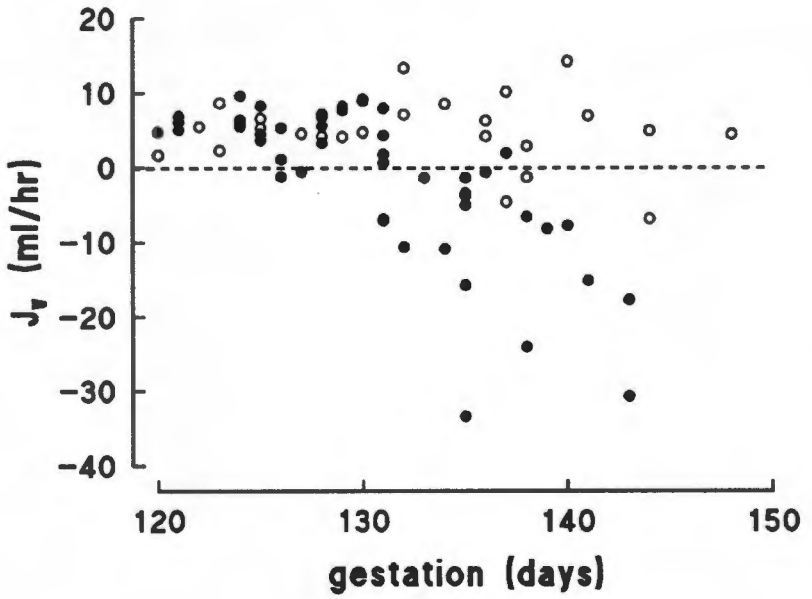
Gestation (days)	n		J_v.control (ml/h)		J_v.adren (ml/h)	
	Non-Tx	Tx	Non-Tx	Tx	Non-Tx	Tx
120-124	9	5	11.7 (±1.0)	5.9* (±1.4)	6.5 (±0.6)	4.7 (±1.3)
125-129	12	6	13.2 (±1.3)	8.3* (±1.0)	4.5 (±1.0)	4.9 (±0.4)
130-134	10	6	17.3 (±2.0)	16.0 (±3.9)	-1.3 (±2.4)	9.3* (±1.4)
135-139	11	5	17.0 (±1.5)	10.5 (±2.5)	-9.2 (±3.3)	1.9* (±2.4)
140+	4	5	10.3 (±2.8)	14.3 (±1.4)	-17.9 (±4.8)	3.7* (±4.0)

* significantly different from Non-Tx group ($p < 0.05$)

The most striking difference between the two groups however was in their response to adrenaline. In non-Tx fetuses from all gestation groups infusion of adrenaline produced a significant slowing of basal secretion rates. However, only in fetuses older than 130 days gestation was secretion brought to a halt and reabsorption of lung liquid observed. This reabsorptive response increased considerably with advancing gestational age. In Tx fetuses, all age groups above 125 days exhibited a small significant fall in basal secretion rates during adrenaline infusion, but, in contrast to the non-Tx fetuses, reabsorption was observed in only three out of the seventeen Tx fetuses (18%) older than 130 days gestation. The reabsorption rates in these 3 Tx fetuses were considerably less than expected for euthyroid fetuses of the same gestations. In the non-Tx group, reabsorption was seen in eighteen of twenty-four

(72%) fetuses older than 130 days gestation (fig 1).

Figure 1 *Secretion or absorption rates during adrenaline infusion in Tx and non-Tx fetuses.*



Ordinate: J_v lung liquid secretion rate during i.v. adrenaline infusion at 0.5 ug/min. Negative values indicate absorption. Abscissa: gestational age at time of experiment. ● euthyroid (non-Tx) fetuses; O thyroidectomised (Tx) fetuses.

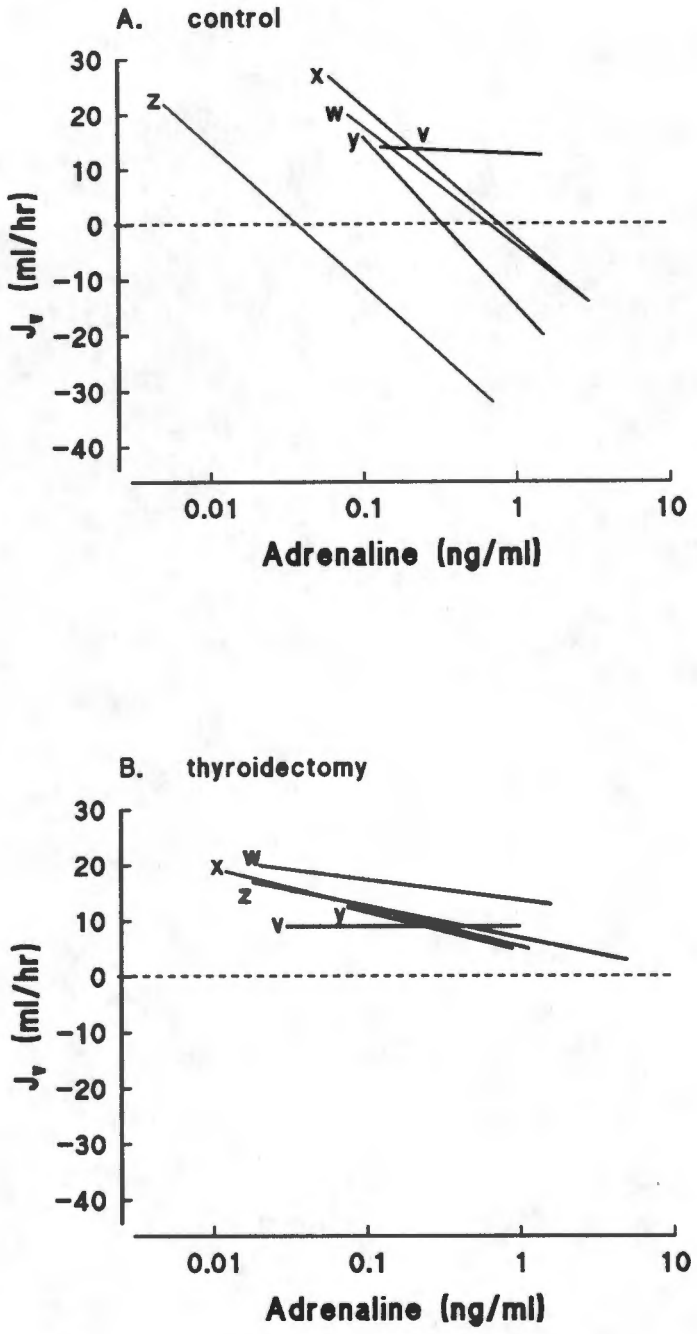
In the one Tx fetus with evidence of thyroid regeneration the responsiveness to adrenaline resembled that of the euthyroid rather than the hypothyroid group. Thus, at 144 days, the secretion rate in this fetus changed from 17 ml/h during the control period to reabsorption at 21 ml/h during the adrenaline infusion.

Relationship of secretion and absorption rates to plasma adrenaline concentrations.

The mean plasma concentrations of adrenaline in carotid areterial blood before and during adrenaline infusions were not significantly different in the Tx and non-Tx fetuses. Before infusion, the adrenaline concentration (ng/ml) in Tx fetuses was 0.09 ($\pm$0.03, n = 23) ng/ml and in the non-Tx fetuses was 0.10 ($\pm$0.01, n = 32) ng/ml. During adrenaline infusion the adrenaline concentration rose to 1.61 ($\pm$0.28, n = 25) in the Tx fetuses and to 1.01 ($\pm$0.14, n=18) in non-Tx fetuses.

In non-Tx fetuses over 130 days gestation, Brown *et al* (1983) demonstrated dependence of secretion rate (J_v) on plasma adrenaline concentration [A], and as gestation advanced this dependence increased strikingly, as shown by gestational changes and the intercept and slope of the regressions of J_v on ln[A] (fig 2, table 2). In the thyroidectomised fetuses the dependence of J_v on ln[A] was much less and showed little or no change with gestational age.

Figure 2. *Regressions of J_v on Ln[A] at five gestations.*



"v"= 129-131 days, "w"= 132-134 days, "x"= 135-137 days, "y"= 138-140 days, "z"= 141+ days. Fig 2A, regressions for non-Tx fetuses calculated from data of Brown et al. (1983). Fig 2B, regressions for Tx fetuses.

Table 3. Parameters of the regression $J_v = a + b.ln[A]$ (see fig.2) for Tx and non-Tx fetuses for the different gestational age groups. $[A_I]$ (ng/ml) is the intercept of regression of $J_v = 0$. For the Tx fetuses this value for obtained by extrapolation of the regressions x,y and z from Fig2.

Gestation (days)		n	a	b	$[A_I]$(ng/ml)
129-131 (v)	Tx	6	-10.4(±5.3)	0.6(±0.9)*	-
	Non-Tx	8	-17.6(±10.8)	0.8(±2.0)*	-
132-134 (w)	Tx	6	-11.8(±2.6)	2.0(±1.1)	-
	Non-Tx	11	7.0(±2.6)	8.4(±1.1)	0.43
135-137 (x)	Tx	7	-4.6(±2.3)	3.3(±1.1)*	4.03
	Non-Tx	11	6.5(±3.6)	9.1(±1.9)	0.49
138-140 (y)	Tx	4	-5.7(±5.7)	2.8(±3.2)	7.66
	Non-Tx	10	17.5(±5.1)	11.5(±2.7)	0.22
140+ (z)	Tx	6	-6.3(±2.6)	2.7(±1.2)*	10.31
	Non-Tx	4	42.5(±9.3)	11.1(±2.4)	0.03

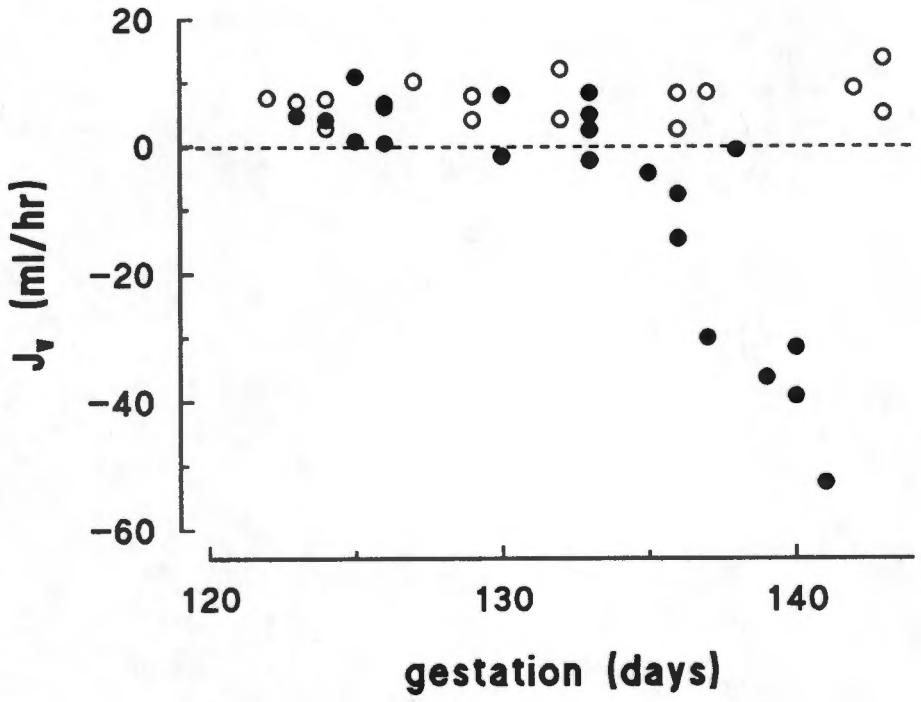
In the euthyroid (non-Tx) fetuses an estimate of the minimum concentration required to induce absorption $[A_I]$ was obtainable from the intercept of the regression of J_v on ln[A] at $J_v = 0$ and this decreased from 0.43 to 0.029 ng/ml during the gestaton period between 132 and 144 days. In the Tx fetuses, estimates of $[A_I]$ could be made only by extrapolation of the regressions. When this was carried out on the regressions shown as *x,y and z* in fig 2.$[A_I]$ values between one and two orders of magnitude greater in the Tx fetuses than in the non-Tx fetuses were obtained (see fig 2 legend).

The effect of dibutyryl cyclic AMP (db-cAMP) added to lung liquid.

It has been shown that reabsorption of lung liquid can induced by adding db-cAMP directly to lung liquid and that this effect increases with gestation in close parallel to the maturation of the response to adrenaline (Olver *et al*, 1987). To test this response after thyroidectomy, we carried out 18 experiments on 5 thyoidectomised fetal sheep to measure the effect of adding 5mg db-cAMP to lung liquid to give an estimated initial concentration of 10^{-4}M. It had been shown by Olver *et al* (1987) that the maximal effect of db-cAMP took place in the second hour after putting this substance into lung liquid. For this reason the effect on J_v was measured at 90 - 120 min after adding db-cAMP.

As shown in Fig 3, which compares results in the Tx fetuses with those in the non-Tx fetuses reported by Olver *et al* (1987), no increase with gestation in the response to db-cAMP was seen in the Tx fetuses.

Figure 3 *The effect of db-cAMP on lung liquid secretion in Tx and non-Tx fetuses*



Secretion (J_V) or absorption rate ($-J_V$), 90-120 min after adding db-cAMP (10^{-4}M) to lung liquid vs gestational age at time of experiment. ● *- euthyroid (non-Tx) fetuses; o - thyroidectomised fetuses.*

Chapter 4

THE INFLUENCE OF T_3 AND T_4 ON MATURATION OF THE ADRENALINE-SENSITIVE LUNG LIQUID REABSORPTIVE MECHANISM.

Having shown that maturation of the reabsorptive response to both adrenaline and db-cAMP is severely inhibited by removing the fetal thyroid gland at 118 days we sought to confirm the role of thyroid hormones in this maturational process and to assess the relative importance of thyroxine (T_4) and its active metabolite tri-iodothyronine (T_3). In this part of the study these hormones were infused into hypothyroid (thyroidectomised) fetuses, commencing infusion at 110, 118, 125 or 131 days gestation and continuing until the fetuses delivered. We compared maturation of the responsiveness to adrenaline in these fetuses with that taking place in euthyroid and hypothyroid fetuses.

In-utero fetal thyroidectomy and catheterisation were performed on the fetuses of 16 pregnant ewes. In fetuses receiving hormones from 110 and 118 days, surgery was performed 3 to 4 days before T_3 or T_4 infusion was commenced, i.e surgery at 106-107 days in 3 fetuses infused with T_3 from 110 days; surgery at 114-115 days in fetuses infused with T_3 (3 fetuses) or T_4 (4 fetuses) from 118 days. In fetuses receiving T_3 from 125 days (3 fetuses) or 131 days (3 fetuses), surgery was performed at 118 days which resulted in a longer period of hypothyroidism before hormone infusion was commenced.

Experiments were conducted at 3 to 8 day intervals following the commencement of thyroid

hormone infusion (n = 60). In each case T_3 or T_4 was infused continuously until the fetus delivered spontaneously or the catheters became blocked. T_3 was infused at 60 ug/day in the experiments started at 110 and 118 days gestation and at 120 ug/day from 125 and 131 days gestation. T_4 was infused at 50 ug/day in those started at 118 days. The higher dose of T_3 was chosen to take account of the larger body mass of the older fetuses. The higher infusion rates, however, resulted in considerably higher T_3 levels than expected (*see below*).

Adrenaline levels

There was a significant rise in resting fetal plasma adrenaline levels during adrenaline infusion at 0.5 ug/min (0.05 ± 0.01 ng/ml to 0.93 ± 0.04 ng/ml, n = 38). This rise in concentration was similar to that seen during adrenaline infusion in hypothyroid and euthyroid fetuses (see chapter 3).

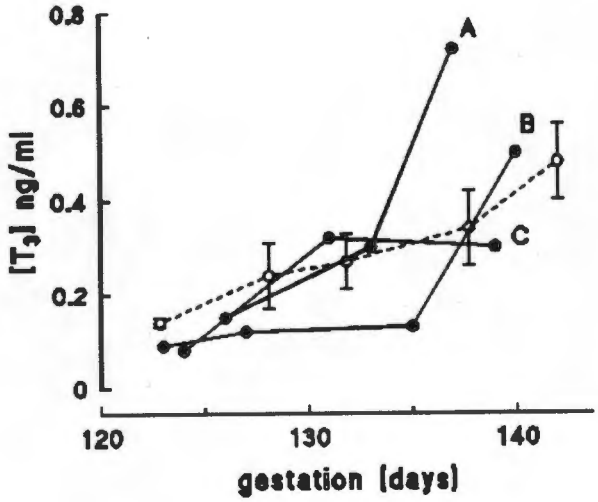
Thyroid hormone levels: Infusion of T_4. Continuous intravenous infusion of T_4 (50 ug/day) was started 2 or 3 days after thyroidectomy at 114-117 days gestation. At the time of starting the infusions the mean fetal plasma T_4 level was 19.3 ng/ml (range 3.9 - 42.7 ng/ml, n = 4) and plasma T_3 levels were at or below the lower limit of detection of the assay (0.09 ng/ml). Following hormone infusion mean plasma T_4 concentration rose to 77.7 ng/ml (s.e.m. 7.7 ng/ml, n = 13). The rise in plasma T_3 concentrations in these T_4 infused fetuses during the infusion period was similar to that seen in non-thyroidectomised fetuses (fig 1).

T_3 data from only 3 of the four T_4-infused fetuses is shown as the arterial sampling catheter

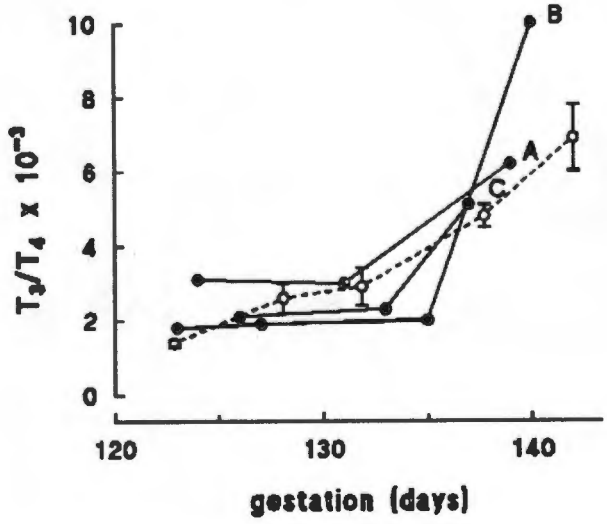
in one of these fetuses became blocked at 131 days. Since these fetuses had been thyroidectomised prior to infusion, the increasing levels of T_3 in fetal plasma were presumed to reflect increasing peripheral ß-monodeiodination of T_4 in the fetus. This deiodination activity, expressed as the ratio of T_3 to T_4 concentration at different gestational ages (T_3/T_4), showed a progressive increase with gestation similar to that seen in the euthyroid fetuses of similar gestations (fig 1). The small fall in T_3 levels in one of the T_4 infused fetuses (**fetus C**) towards the end of the period of study was attributed to a relatively large fall in T_4 concentration in this fetus as its T_3/T_4 ratio increased in the expected way during this time.

Figure 1. *T_3 levels in fetal plasma (panel A) and T_3/T_4 ratio during T_4 infusion to thyroidectomised fetal sheep (panel B).*

A.



B.



● - values from 3 fetuses (A,B,C) thyroidectomised at 114 - 117 days and infused with T_4 (50 ug/day), starting either 2 or 3 days later and continuing until delivery or death of the fetus. o - mean values (± s.e.m.) from 8 non-thyroidectomised fetuses in 5-day gestational age groups. (from Brown et al, 1983)

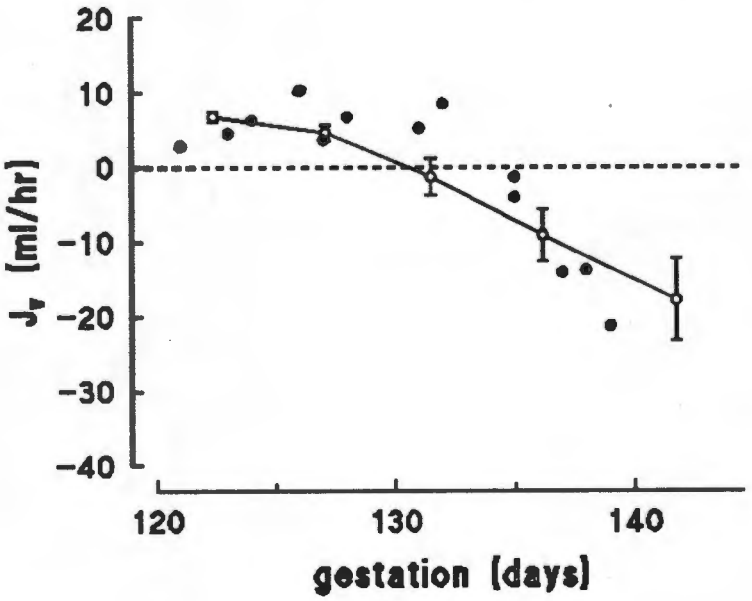
Infusion of T_3. By the time T_3 infusion was started fetal plasma T_3 levels had fallen below the limits of detection of the assay in all fetuses in this group. After starting the infusion the fetal plasma T_3 concentration rose to a stable level by the third day of infusion. In fetuses where the T_3 infusion rate was 60 ug/day, plasma T_3 concentration rose to a mean of 0.87 ng/ml (s.e.m. 0.10 ng/ml, n = 15), while those infused at the higher rate of 120 ug/day had mean T_3 levels of 2.78 ng/ml (s.e.m. 0.31 ng/ml, n = 27).

Effect on Heart Rate, Blood Pressure, and Arterial Blood Gas Tensions. During adrenaline infusion there was a significant rise both in heart rate, (T_3-infused group: 153$\pm$5 to 169$\pm$4 beats/min, n = 14, T_4-infused group 158$\pm$5 to 177$\pm$10 beats/min, n = 10) and mean blood pressure (T3 group: a rise of 5.9$\pm$0.8 mm/Hg, n = 22, T_4 group: a rise of 4.2$\pm$1.6 mm/Hg, n = 9). These changes were very similar to those reported for control (Brown et al, 1983) and thyroidectomised fetuses (Chapter 3). There were no significant differences in the responses of fetuses infused with 60 or 120 ug T_3 per day. Arterial blood gas tensions before and during adrenaline infusion were unchanged in all groups.

Maturation of the lung liquid reabsorptive response in T_4-infused fetuses. Infusion of T_4 at 50 ug/day in 4 fetuses from 118 days following thyroidectomy at 114-117 days restored the maturation of the reabsorptive response to adrenaline which had been inhibited in the hypothyroid fetuses (fig 2). The gestation at which the reabsorptive response was first seen may have been delayed slightly when compared to the control data but the magnitude of the response was the same in both groups within all the gestational age groups (table 1). There

was no apparent dependence of reabsorptive capacity on the absolute concentration of T_4 or T_3 in these fetuses. Thus fetus "C" in fig. 1 had the largest reabsorptive response to adrenaline (-21.3 ml/hr) of the mature T_4-infused fetuses whilst its plasma T_3 and T_4 levels were the lowest in the group.

Figure 2 *Lung liquid secretion and absorption rates during 30 minute periods of adrenaline infusion (0.5 ug/min) to thyroidectomised fetuses infused with* T_4.



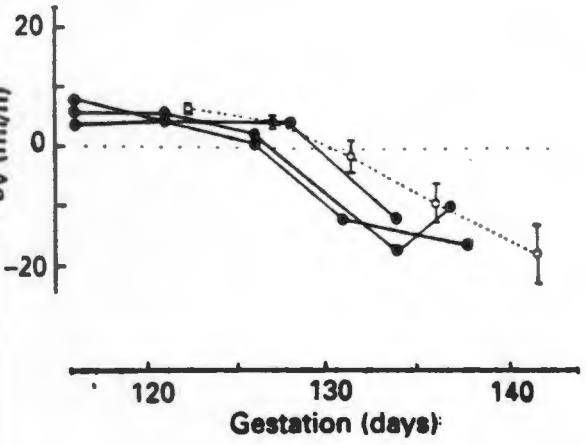
Thyroidectomy was performed at 114-117 days and infusion with T_4 *(50 ug/day) started 2 or 3 days later.* ● *- values from 4 thyroidectomised,* T_4*-infused fetuses. o - mean values (± s.e.m) from 25 euthyroid, non-thyroidectomised fetuses (Brown et al, 1983) in 5-day gestational age groups. Values for the pre-adrenaline infusion secretion rates are not shown here.*

Maturation of the lung liquid reabsorptive response in T_3-infused fetuses. When the T_3 infusion was started at 110 days, the reabsorptive response to adrenaline was first seen after 130 days gestation, some 3 weeks after the infusion had commenced. The magnitude of the response to adrenaline infusion before 135 days was significantly greater than in the control group (table 1) and the earliest gestation at which the reabsorptive response appeared may have been brought forward by a few days as a result of the T_3 infusion (fig 3). T_3 supplementation from 118 days resulted in a pattern of maturation very similar to the T_4-infused and control groups, i.e. the reabsorptive response to adrenaline infusion was seen from 130 days onwards (fig 3 and table 1).

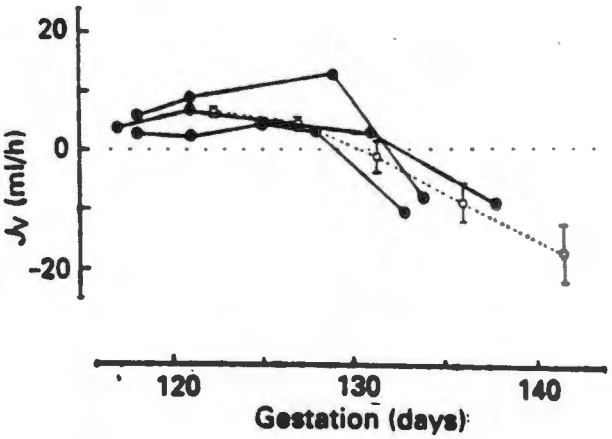
In the 3 fetuses infused with T_3 (120 ug/day) from 125 days, the capacity to reabsorb lung liquid was delayed until 135 days gestation - some 10 days after the T_3 infusion had commenced and about 5 days later than is usual in the euthyroid fetuses (fig 3 and table 1). After this gestation the magnitude of the response to adrenaline infusion was not significantly different from that of the control group. The 3 fetuses infused with T_3 (120 ug/day) from 131 days experienced a similar delay in the appearance of the reabsorptive response (fig 3) with reabsorption of lung liquid being seen in this group only after 138 -141 days gestation. The magnitude of the response after this gestation was similar to that seen in the control group.

FIG. 3. *Lung liquid secretion and absorption rates during 30 minute periods of adrenaline infusion at 0.5 ug/min to thyroidectomised fetal sheep infused continuously with T_3.*

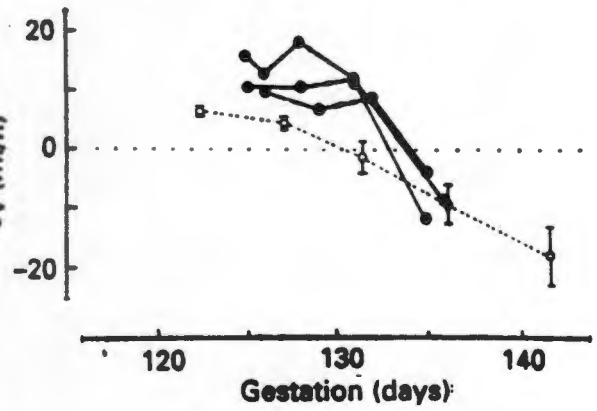
A: T_3 from 110d



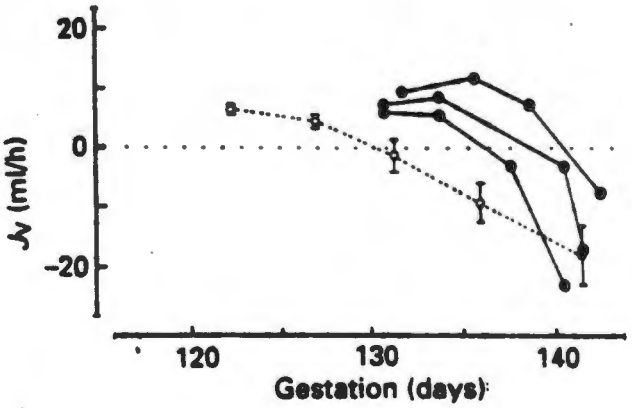
B: T_3 from 118d



C: T_3 from 125d



C: T_3 from 131d



In each panel: ● ● : secretion or absorption rates during adrenaline infusion in T_3-infused fetuses. O O : mean values (±s.e.m.) for non-thyroidectomised fetuses (from Brown et al, 1983) in 5-day gestational age groups.

TABLE 1 *Mean (±s.e.m.) secretion (J_V) or absorption rates ($-J_V$), ml/hr, of fetal lung liquid during i.v. adrenaline infusion (0.5 ug/min) in thyroidectomised fetal sheep following T_3 or T_4 supplementation. T_3 was infused continuously at 60 ug/day in fetuses in which infusions started at 110 days (group A) or 118 days (group B), and at 120 ug/day in fetuses in which infusions were commenced at 125 (group C) or 131 days (group D). T_4 was infused continuously in 3 fetuses at a rate of 50 ug/day. Non-Tx data from Brown et al (1983).*

J_v (ml/h)

Gestional oup (days)	T_3 (from day below) 110	118	125	131	T_4 (from day 118)	Control (non-Tx)
115–119	6·3±0·7	—	—	—	—	4·0±0·9
n	3	—	—	—	—	3
120–124	4·6±0·4	5·9±1·6	—	—	4·5±1·2	6·5±0·6
n	3	3	—	—	3	9
125–129	2·3±1·0	6·7±2·7	11·5±3·9*	—	6·8±2·4	4·5±1·0
n	3	3	3	—	3	12
130–134	−13·3±1·5*	−5·4±3·6	10·5±0·9*	11·1±3·8*	6·9	1·3±2·4
n	3	3	3	3	2	10
135–139	−13·3	—	−15·7±6·8	5·9±2·0	−11·0±4·1	−9·2±3·3
n	2	—	4	6	5	11
140–144	—	—	—	−10·7±3·5	—	−17·9±4·8
n	—	—	—	5	—	4

* significantly different from control values

Resting secretion rates in control, thyroidectomised and T_3 treated fetuses.

Over the period of study (120 to 147 days gestation) resting secretion rates in control fetuses show a small rise between 130 and 140 days gestation before falling back to the levels seen at around 120 days (Brown et al, 1983). In the Tx fetuses resting secretion rates were significantly lower than in the control group below 130 days gestation, but these were restored in the T_3-infused fetuses whose secretion rates were similar to control values in all gestational age groups. The T_4-infused fetuses had resting secretion rates which were not different from control values except in the 120-124 day age group which had lower values.

Table 2. *Comparison of mean (±s.e.m.) resting secretion rates, J_v (ml/hr), between control (Con), thyroidectomised (Tx), T_3 infused (60 ug/day) from 110/118 days (T_3), or T_4 infused (50 ug/day) from 118 days (T4) fetuses.*

Gest. Group (days)		J_v (ml/hr) Con	Tx	T3	T4
120 - 124	J_v	11.5	5.9*	9.1	7.4*
	s.e.m.	(0.8)	(1.4)	(1.2)	(0.8)
	n	9	5	7	3
125 - 129	J_v	13.2	8.3*	15.0	13.3
	s.e.m.	(1.3)	(1.0)	(3.2)	(2.6)
	n	11	6	6	3
130 - 134	J_v	17.2	16.0	13.1	15.3
	s.e.m.	(1.9)	(3.6)	(3.4)	(2.6)
	n	10	6	6	3
135 - 139	J_v	17.0	10.5*	-	18.2
	s.e.m.	(1.3)	(2.3)		(3.2)
	n	11	6		4
140±	J_v	10.3	14.3	-	-
	s.e.m.	(2.5)	(1.8)		
	n	4	5		

* significantly different from control value on unpaired T testing ($p < 0.05$)

Chapter 5

SYNERGISM OF T_3 AND HYDROCORTISONE IN EARLY MATURATION OF THE REABSORPTIVE RESPONSE TO ADRENALINE.

Infusion of T_3 or T_4 into fetuses thyroidectomised at 118 days allowed a normal evolution of the reabsorptive response to adrenaline from around 130 days. However, our failure to induce an early appearance of the reabsorptive response by precocious exposure of the fetus to T_3 suggested that some other factor must play a role in determining its maturation during gestation. Steroid hormones, alone or in combination with thyroid hormones, are known to be important in determining the maturation of structural aspects of the lung and its surfactant system in the fetus. Injection of fetal rabbits with dexamethasone at 21 days (glandular stage of development) resulted, when examined 36 hours later, in more airway branching and at 26 days hormone treatment induced lammelar body formation and loss of glycogen (Kikkawa, 1971). Infusion of dexamethasone in fetal sheep resulted in thinning of the alveolar septae and increase in alveolar size (Platzker 1975). The mechanism of glucocorticoid effects on morphogenesis is not known. An addititive or permissive effect of glucocorticoid hormones on maturation induced by T_4, of cytoplasmic organelles was demonstrated in fetal rabbit lung (Hitchcock, 1979). An additive effect of increased choline incorporation into phosphatidyl has been reported with T_3 and corticosteroids both in vivo and in fetal lung explant culture (Gross, 1984; Ballard, 1984).The following experiments were undertaken to examine the influence of hydrocortisone, alone, and in combination with T_3, on the maturation of the reabsorptive response. Thyroidectomy and catheterisation were

performed *in-utero* on 9 fetuses at 112-115 days gestation.

T_3 and cortisol levels.

At the time of the first experiment (2-4 days after surgery) fetal plasma T_4 and T_3 concentrations had fallen to very low levels (mean T_4 14.8 $\pm$ 2.1 ng/ml, mean T_3 0.12$\pm$0.03 ng/ml) as a result of fetal thyroidectomy (cf values for euthyroid fetuses of 120-130 days gestation; T_4 98.8 ng/ml, T_3 0.21 ng/ml). Following 3 days of T_3 or combined T_3 & hydrocortisone infusion mean T_3 concentration had risen to 0.78 $\pm$ 0.27 ng/ml. Three days after hydrocortisone or T_3 & hydrocortisone infusion plasma cortisol concentrations had risen from 1.04 $\pm$ 0.3 ug/dl to 10.8 $\pm$ 5.1 ug/dl. These concentrations of both T_3 and hydrocortisone were similar to those normally seen in the fetal sheep a few days before delivery (Wu et al, 1978, Fisher et al, 1977).

Fetal blood pressure, heart rate and arterial blood gas monitoring.

All 3 experimental groups of fetuses showed similar rises in mean blood pressure and heart rate during adrenaline infusion both before and after exposure to hormones. Thus the increases during adrenaline infusion before hormone administration were: blood pressure 4.2$\pm$1.2 mm/Hg, heart rate 13.3$\pm$7.7 beats/min, whereas after 3 days of hormone exposure these increases were 3.6$\pm$1.2 mmHg and 9.5$\pm$4.1 beats/min. Adrenaline infusion caused a small fall in Ph (7.39$\pm$0.01 to 7.37$\pm$0.01, $p < 0.05$) and a small rise in pCO_2 (45.7$\pm$1.7 to 48.4$\pm$1.8, $p < 0.05$). There were no differences in these measurements between the 3 groups and no changes in Po_2 were detected.

The effect of T_3 and hydrocortisone on the response of lung liquid secretion/absorption to adrenaline.

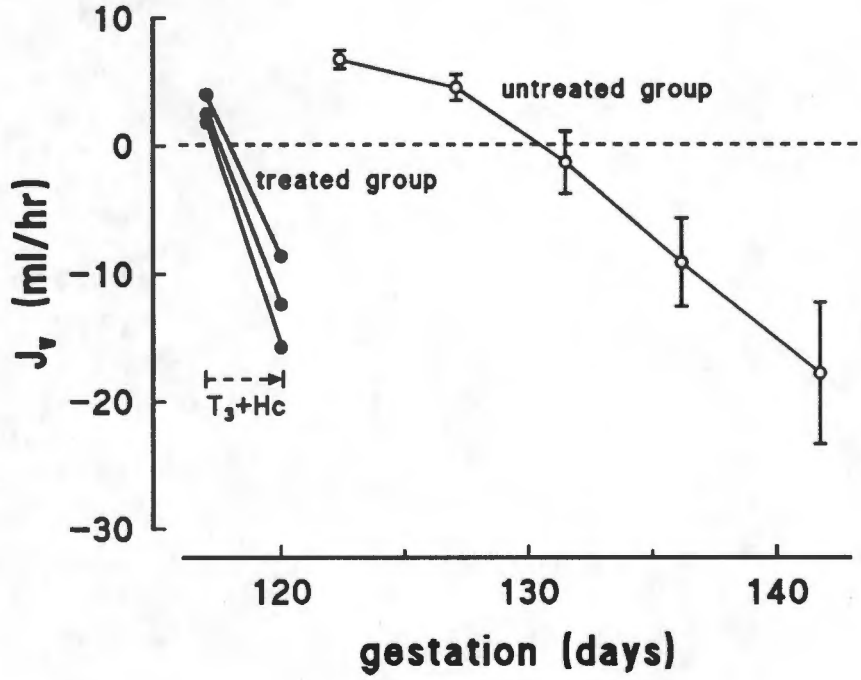
In all 3 groups of fetuses, after 3 days of hormone exposure, there was a similar rise in resting (pre-adrenaline infusion) secretion rate (6.2±0.5 ml/hr to 9.1±1.3 ml/hr, n = 9, $p < 0.05$). In all 3 groups the control adrenaline infusion before T_3, hydrocortisone or combined hormone exposure produced the small, expected decrease in secretion rate normally seen at this gestation.

TABLE. *Mean (±s.e.m.) secretion (+) or absorption (-) rates (ml/hr) of fetal lung liquid before (Jv_c) and during (Jv_a) adrenaline infusion (0.5 ug/min) in 9 thyroidectomised fetal sheep in experiments performed before and 3 days after being administered T_3, hydrocortisone or T_3 & Hc.*

	before hormone		after hormone	
	Jv_c	Jv_a	Jv_c	Jv_a
T_3-infused group:	+5.8	+2.8	+7.1	+2.4
	+8.6	+5.7	+14.4	+8.6
	+7.1	+3.5	+6.2	+6.8
Hc-infused group:	+5.8	+7.4	+14.3	+6.6
	+7.2	+5.9	+9.5	+3.8
	+5.1	+1.2	+5.5	+2.9
T_3 & Hc-infused group:	+7.1	+2.5	+11.1	-15.8
	+2.8	+1.8	+4.3	-12.4
	+4.0	+4.0	+4.3	-8.6

In both the T_3 and hydrocortisone infused groups the secretion rates during adrenaline infusion in the second experiments, after 3 days hormone exposure, were no different from those in the first pre-hormone experiment. However, in the 3 fetuses treated with a combination of T_3 and hydrocortisone there was a most striking reabsorptive response to adrenaline at 120 days gestation, comparable to that normally seen in the euthyroid fetus at 135 to 140 days.

Figure *Lung liquid secretion rate (Jv) or reabsorption rate (-Jv) during intravenous adrenaline infusion (0.5 ug/min) plotted against gestational age (days). The resting secretion rates before adrenaline infusion are not shown.*



● - *values from three thyroidectomised fetal sheep before (116 days) and 3 days after (119 days) continuous infusion of* T_3 *(60 ug/day) and hydrocortisone (10 mg/day).*

o - *mean (* ±*s.e.m.) Jv for 25 non-thyroidectomised fetuses grouped in 5-day gestations from 120 to 145 days (from Brown et al, 1983).*

CHAPTER 6

T_3 AND HYDROCORTISONE SYNERGY IN EARLY MATURATION OF THE REABSORPTIVE MECHANISM : TIME COURSE, DOSE-RESPONSE AND INHIBITION WITH CYCLOHEXAMIDE.

The following experiments were carried out to determine the duration of hormone action required to induce a reabsorptive response, to determine whether the response was reversible on stopping hormone administration, and also to determine the effect on this hormonal action of cyclohexamide, an inhibitor of protein synthesis, when applied to the pulmonary epithelium by addition to lung liquid.

A total of 37 experiments were performed on 12 fetuses which had undergone *in-utero* thyroidectomy and catheterisation at 111 to 115 days gestation.

Hormone concentrations. Fetal plasma levels of T_3 at the start of hormone replacement (116 days) were close to or below the lower limit of detection of the assay (0.07 ng/ml). In the fetuses where T_3 was infused continuously (60 ug/day), the mean plasma T_3 level after 24 hours was 0.70 ng/ml (s.e.m. $\pm$0.15, n = 3, and after 72 hours was 1.59 ng/ml (s.e.m. $\pm$0.77, n = 4).

In each of the 3 experiments in which the fetus was given a bolus of T_3 the plasma T_3 concentration fell rapidly and had returned to the baseline at 24 hours. Intermediate concentrations measured at 27-53 min, 153-198 min and 296-303 min allowed the approximate half-life of T_3 in fetal plasma to be calculated. In all cases where T_3 infusion was discontinued, the plasma concentration at the start of re-infusion experiments (24-48 hours later) was at or below the limits of detection.

Plasma cortisol levels at 116 - 118 days (before the start of hormone infusion) were low (mean 0.6 ± 0.2 ug/100 ml, n = 11) and were similar to normal values reported for fetal sheep of this gestation (Ballard et al, 1982). In the fetuses infused with cortisol 10 mg/day for 3 days, mean plasma concentration of this hormone at 24 hours was 4.3 ± 0.4 ug/100 ml, n = 3, and at 72 hours was 4.2 ± 1.3 ug/hr, n = 4. Injection of a bolus of cortisol (2 mg) followed by infusion (10 mg/day) in the shorter experiments gave similar mean plasma levels of cortisol at 2 hours (4.7 ± 0.6 ug/100 ml, n = 3), 4 hours (3.1 ± 0.6 ug/100 ml, n = 6) and 24 hours (3.7 ± 1.0 ug/100 ml, n = 4). Following discontinuation of hydrocortisone infusion for 24 - 48 hours, a low level of mean plasma cortisol levels measured before re-infusion experiments (0.78 ± 0.19 ug/100 ml) showed that the infused hydrocortisone had been cleared from the fetal circulation.

Time-course of action of T_3 & Hydrocortisone.

Bolus T_3 and continuous hydrocortisone infusion : response to adrenaline at 4 and 24 hours.

Four thyroidectomised fetuses at 116-118 days gestation were given T_3 (30 ug bolus i.v.) and hydrocortisone (2 mg bolus plus 10 mg infused over 24 hours) by the intravenous route. The effect of one-hour intravenous adrenaline infusion (0.5 ug/min) on lung liquid secretion was measured before administering T_3 and hydrocortisone and again after 4 and 24 hours. In the control period before giving T_3 and hydrocortisone, adrenaline infusion had no effect on mean Jv (before adrenaline J_v = 5.8$\pm$2.1 ml/hr; during adrenaline J_v = 5.3$\pm$1.2 ml/hr). Four hours after exposure to T_3 and hydrocortisone reabsorption took place in 2 of the 4 fetuses (mean -0.5$\pm$1.64 ml/hr, range -4.0 to +2.8 ml/hr) during adrenaline infusion. Twenty-four hours after starting the hormones reabsorptive effect was present in all 4 fetuses (mean -3.6$\pm$2.2 ml/hr, range -0.9 to -7.0 ml/hr).

Continuous T_3 and hydrocortisone infusion : effect of exposure to adrenaline at 24 and 72 hours. A further 5 thyroidectomised fetuses were infused continuously from 116 days for 1 to 3 days with T_3 (60 ug/day) and hydrocortisone (10 mg). Twenty four hours after starting the hormones 3 fetuses, infused with adrenaline, showed a reabsorptive response (mean -4.4$\pm$0.6 ml/hr) similar to that seen in the above experiments. After 72 hours T_3 and hydrocortisone infusion, the reabsorptive response was significantly greater than after 24 hours (mean -11.1$\pm$2.2 ml/hr, $p < 0.05$, unpaired t test).

Figure 1 *Prolonged adrenaline infusion: example experiment*

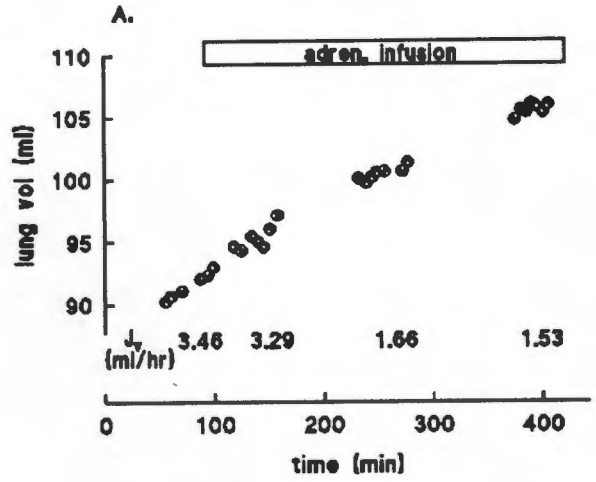
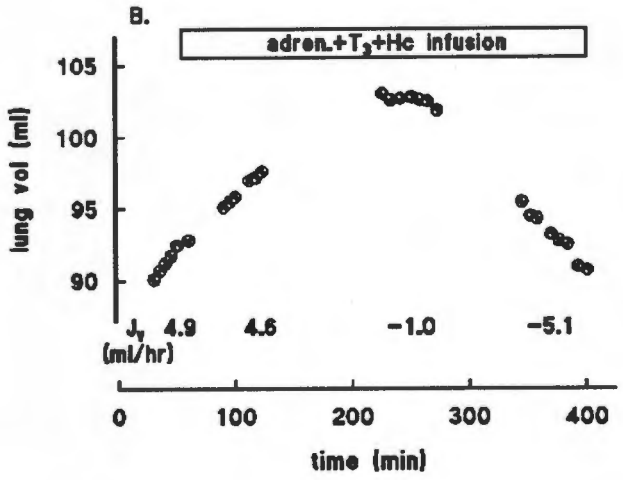


Figure 1b

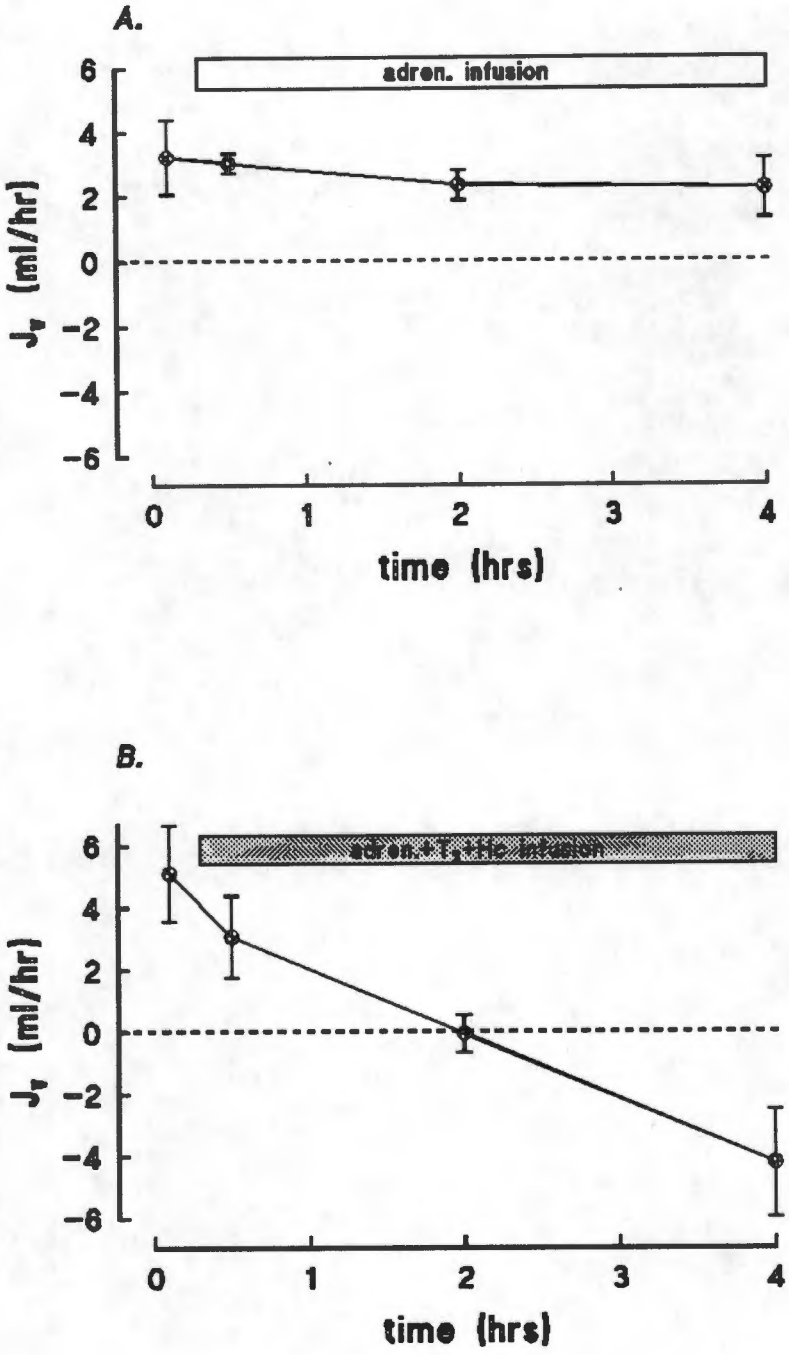


Accumulated lung liquid volume plotted against experimental time. Each point represents one lung liquid sample. Panel A: *Secretion or absorption rates for periods of sampling before and during prolonged adrenaline infusion.* Panel B: *Similar experiment conducted on the same fetus one day later.* T_3 *and hydrocortisone infused concurrently with adrenaline.*

Prolonged infusion of adrenaline for 5 hours. Since the effect of T_3 and hydrocortisone on the reabsorptive response to adrenaline was already evident 4 hours after giving these hormones, an attempt was made to define further its timing by measuring the response to adrenaline infused continuously at 0.5 ug/min for a 4 to 5 hour period.

The experiments were performed on 2 successive days on 3 thyroidectomised fetuses starting at 115-118 days gestation. On the first day, the effect on J_v was measured during 4 to 5 hours continuous infusion of adrenaline without hormone administration; and on the second day the same adrenaline infusion was performed, but, in this case, following intravenous administration of T_3 (30 ug bolus) and hydrocortisone (2 mg bolus, then infusion at 0.42 mg/h).

Figure 2. Prolonged adrenaline infusion: mean values of secretion and absorption rates.



means (± s.e.m.) of secretion rate or absorption rate during adrenaline infusion at 0.5 ug/min to 3 fetuses of 115-118d gestation in experiments performed over 2 successive days.

Panel A: experiments without T_3 and hydrocortisone.
Panel B: experiments conducted the following day with T_3 and hydrocortisone

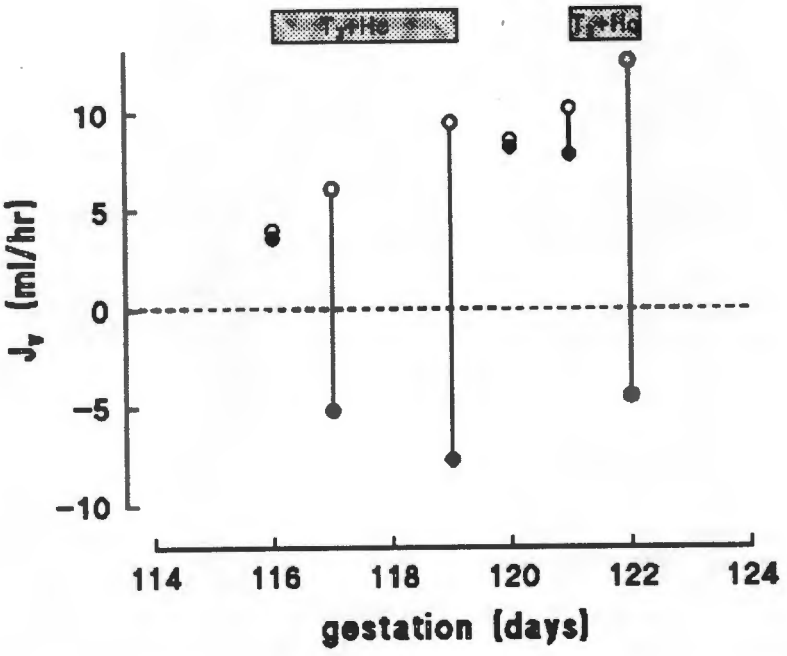
On the first day, without T_3 and hydrocortisone infusion, only a small fall in J_v was observed (fig 2) whereas on the second day, following combined administration, a striking effect on J_v was observed (mean J_v = -4.23$\pm$2.5 ml/hr).

Reversibility of the combined T_3 and hydrocortisone effect.

The reversibility of the reabsorptive response was tested in 4 fetuses given T_3 (30 ug as a bolus) and hydrocortisone (2 mg bolus and then 10 mg/day) and also in 3 fetuses given continuous T_3 (60 ug/day) and hydrocortisone (10 mg/day).

The effect of adrenaline infusion on J_v was again tested at 24 hours (3 fetuses) or 48 hours (4 fetuses) after discontinuation of the T_3 and hydrocortisone infusion. In each case it was found that reabsorption no longer took place during adrenaline infusion (mean J_v at 24 hours 9.5+1.5 ml/hr, and at 48 hours 6.9$\pm$2.1 ml/hr). Following this recovery period, re-infusion for 24 hours of T_3 and hydrocortisone in one of these fetuses (fig 3) again induced the reabsorptive response to adrenaline. (Jv = -4.5 ml/hr).

Figure 3. *Reversibility of the effect of T_3 and hydrocortisone*



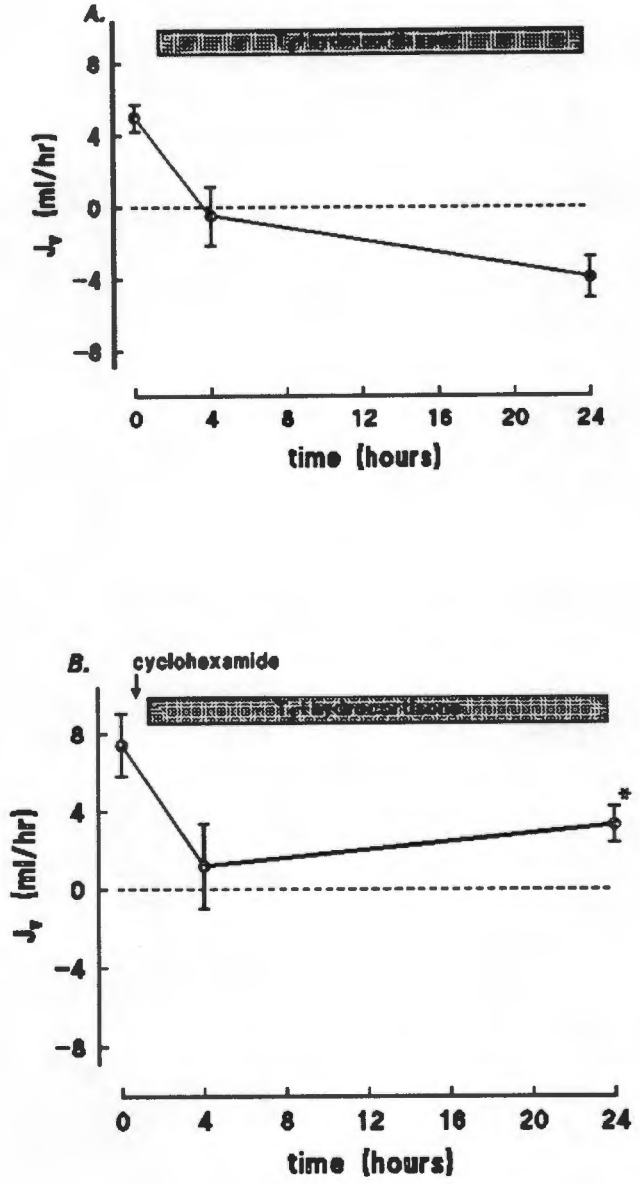
Results from one fetus showing 6 individual experiments measuring resting secretion rates (O) and secretion or absorption rates during adrenaline infusion (●). The vertical lines joining these symbols indicate the fall in secretion rate during the experiment while adrenaline is infused. Periods of infusion with T_3 and hydrocortisone are indicated by the shaded bars.

Effect of cyclohexamide on induction by T_3 and hydrocortisone of the reabsorptive response. In the 4 fetuses referred to in the first section of Results which had shown a reabsorptive response to adrenaline at 24 hours after administration of T_3 and hydrocortisone, further experiments were conducted to test the effect of the protein synthesis inhibitor, Cyclohexamide, applied to the pulmonary epithelium by addition to lung liquid. Experiments at 24-48 hours after stopping T_3 and hydrocortisone showed that there was

no longer a reabsorptive response to adrenaline. After addition of cyclohexamide to lung liquid to a final, estimated concentration of 3×10^{-4} to 4×10^{-5}M, the two hormones, T_3 (30 ug bolus), and hydrocortisone (2 mg bolus & 10 mg/day) were again given intravenously. Measurements of J_v were made at 4 hours and 24 hours and compared with the results in the same fetuses when the hormones were given without cyclohexamide (fig.4).

As shown in the figure below (fig 4), the value of J_v during adrenaline infusion after the recovery period was similar to that obtained before any T_3 or hydrocortisone had been given. However, at 4 hours the induction of the response to adrenaline by these 2 hormones was blunted in the presence of cyclohexamide and the reabsorptive response completely eliminated when J_v was measured at 24 hours (Jv without cyclohexamide = -3.63$\pm$1.46 ml/hr, Jv with cyclohexamide = +3.29$\pm$0.94 ml/hr; $p < 0.01$). In one of these fetuses Jv was again measured 4 days after stopping the hormone re-infusion (no further cyclohexamide was added to lung liquid during this time). This was followed by a third infusion period of T_3 and hydrocortisone at the same doses. The response to adrenaline after the recovery period (J_v = 7.1 ml/hr) and after 4 hours of T_3 and hydrocortisone infusion (J_v = -5.5 ml/hr) was similar to the results obtained in experiments conducted before exposure to cyclohexamide.

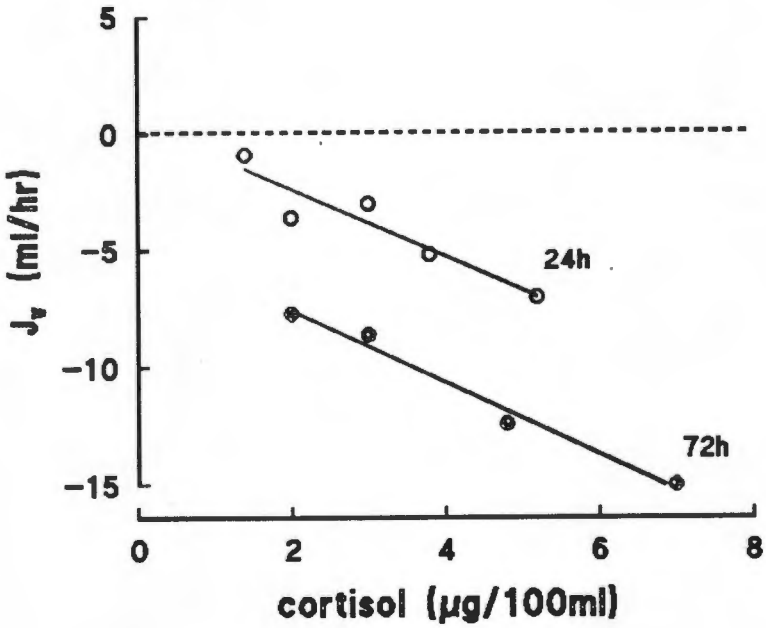
Figure 4. *Effect of cyclohexamide.*



Mean values of secretion (J_V) or absorption ($-J_V$) rate during brief periods of adrenaline infusion before and after exposure to T_3 and hydrocortisone. Panel A: results obtained in the absence of cyclohexamide showing induction of the reabsorptive response at 4 and 24 hours. Panel B. The same experimental protocol in the same 4 fetuses following a 24 hour period of withdrawal of T_3 and hydrocortisone conducted in the presence of cyclohexamide in the lung liquid which prevented the development of a reabsorptive response.

Dependence of response to adrenaline in fetal plasma T_3 and cortisol levels. Results shown in figure 5 show that the absorptive response to adrenaline was greater 72 hours after exposure to the hormones than after 24 hours.

Fig 5. *Dependence of the reabsorptive response on plasma cortisol concentration.*



Lung liquid absorption rates during adrenaline infusion experiments 24h and 72h after commencement of T_3 and hydrocortisone treatment vs fetal plasma [cortisol].

Figure 5 legend (cont.)

Open symbols (O) are values obtained during experiments 24 hours after commencement of T_3 and hydrocortisone infusion. Closed symbols (O) are values obtained following 72 hours of T_3 and hydrocortisone infusion. In each case the reabsorptive response depended on the plasma cortisol concentration.

For the 24 hour values:

$-J_v = -0.461 - 1.448[cortisol] \quad (r=0.947)$

For the 72 hour values:

$-J_v = -4.042 - 1.687[cortisol] \quad (r=0.995)$

The slopes of these lines are not different ($p>0.4$) whereas the intercept of the 72 hr line is significantly lower than that of the 24 hour line ($p<0.001$).

The regression of J_v on ln [cortisol] at 24 hours includes fetuses infused continuously with T_3 (60 ug/day, 2 fetuses) or receiving a bolus of T_3 (30 ug, 3 fetuses) in addition to hydrocortisone. In this case, the dependence of Jv on ln [cortisol] was similar in these fetuses despite major differences in the concentrations of T_3 at the time of the experiment (0.09 ± 0.01 ng/ml in those given T3 30 ug bolus, and 0.68 ng/ml and 1.04 ng/ml in the 2 fetuses given T_3 60 ug continuous infusion). These results and those from experiments conducted following 72 hours of infusion of T_3 60 ug/day, indicate that there is no evident dependence of J_v on fetal plasma T_3 concentration.

CHAPTER 7

DISCUSSION

This investigation stems from 4 important earlier studies from the University \college laboratories in London. Namely, the observation that active Cl^- transport is the driving force for the secretory process of the fetal lung (Olver & Strand, 1974), the identification od ß-agonists as being responsible for the reabsorption of lung liquid during labor and delivery (Walters and Olver 1978), the description of the maturation process that results in the emergence of this reabsorptive capacity in late gestation (Brown et al 1983), and a study which showed that the reabsorptive response was mediated through Na^+ transport from the lumen to the interstitium which could be blocked by amiloride (Olver et al, 1986)

The purpose of the present study was to explore the possible influence of steroid and thyroid hormones on the maturation of ion transport mechanisms of fetal lung ion transport in late gestation. The work was conducted in 3 phases: a study of the effects of thyroidectomy on the evolution of adrenaline-sensitive reabsorptive response, thyroid hormone replacement studies evaluating the importance of T_3 and T_4 to this response, and an investigation of the role and mechanism of steroid hormone interaction in the maturation process. In addition, fetal thyroidectomy and subsequent thyroid hormone and steroid hormone replacement provided the opportunity to study fetal thyroid hormone kinetics.

Fetal thyroid hormone kinetics.

Our data from thyroidectomised fetuses confirm that materno-fetal transfer of thyroid hormones is minimal. Within a few days of thyroidectomy fetal plasma concentrations of T_3 and T_4 had fallen to concentrations which were at or below the limits of detection of our assay, while concentrations of T_4 were $<10\%$ those of the control group. This fall in fetal thyroid hormone concentrations confirmed the observation that the sheep placenta is relatively impermeable to the passage of hormones from the maternal circulation (Comline *et al*, 1970; Hopkins and Thorburn, 1972). The thyroid hormone concentrations in both the thyroidectomised and in the non-thyroidectomised fetuses during the period of study were similar to those found by other investigators (Nathanielz *et al*, 1973; Fisher *et al*, 1977; Nwosu *et al*, 1979; Fraser and Liggins, 1988). Thus, the thyroidectomised fetuses were rendered profoundly hypothyroid for the critical 3 week period at the end of gestation when maturation of the reabsorptive capacity of the lung is normally observed (Brown *et al*, 1983).

Infusion of T_4 into thyroidectomised fetuses from 118 days gestation provided an opportunity to study the ontogeny of ß-monodeiodination of T_4 to T_3 since any T_3 detected will have necessarily been derived from peripheral conversion of infused T_4 rather than from the gland itself. Our thyroidectomised fetuses infused with T_4 showed the normal progressive increase both in the concentrations of T_3 and in the T_3/T_4 ratio during the period of study (118 to 140 days) confirming that the increase in T_3 concentrations usually seen in the fetus at this time results from increasing peripheral deiodination of T_4. The small fall in T_3 concentration seen in one fetus was assumed to be related to a relatively

larger fall in T_4 concentration (fig 1, chapter 4) since the T_3/T_4 ratio in this fetus was similar to that in other infused and normal fetuses of the same gestation.

Infusion of T_4 at 50 ug/day resulted in fetal plasma T_4 concentrations which were similar to those in our full term non-Tx controls and those reported by others (Nathanielsz, Comline, Silver and Thomas, 1973; Fisher *et al*, 1977; Fraser and Liggins, 1988). In fetuses where T_3 was infused at 60 ug/day, fetal plasma T_3 concentrations were about 60 - 100%higher than those seen in euthyroid fetuses at full term whereas fetuses infused at the higher rate of 20 ug/day had mean T_3 concentrations some 5 fold higher than those seen at full term, but similar to those reported for newborn lambs during the postnatal T_3 surge (Sack *et al*, 1976, Nathanielsz *et al*, 1973). The plasma concentrations of T_3 in fetuses receiving continuous infusions of T_3 (60 ug/day) together with hydrocortisone were similar to those measured in fetuses receiving T_3 alone.

The very rapid fall in plasma concentrations of T_3 following bolus injection was an unexpected finding although, since these experiments were done, a report has been published showing that the rate of metabolic breakdown of T_3 is very high in the fetus (Fraser and Liggins, 1988). The T1/2 of T_3 in the human adult is in the region of 24 hours (De Groot, 1982) whereas in our experiments it averaged 68 min.

Fetal cortisol concentrations

Fetal plasma cortisol concentrations are likewise low throughout most of gestation, principally as a result of conversion by the placenta of fetal and maternal cortisol to its inactive metabolite, cortisone (Buster, 1983). A gradual rise in fetal cortisol concentrations is seen in the fetal sheep from about 130 days (Ballard *et al*, 1982). Nathanielsz (1972) reported a later, more pronounced rise in cortisol concentrations to 10 ug/dl only in the last few days before delivery. Thus, in our experiments in which cortisol was infused at 10mg/day, fetuses were exposed precociously (at gestations less than 120 days) to hormone concentrations which are normally seen only 3 to 4 weeks later, close to full term.

Cardiovascular responses to hormone infusion.

The cardiovascular responsiveness to catecholamines does not appear to have been affected by the thyroid status of these fetuses. The resting heart rate and the rise, during adrenaline infusion, in both heart rate and mean blood pressure were very similar in the control, Tx, T_4- and T_3-infused fetuses suggesting that thyroid hormones at the concentrations experienced by these fetuses do not have a direct or indirect influence on cardiac rate. This contrasts with the stimulatory effect of hyperthyroidism on cardiac rate reported in fetal studies which used much higher infusion rates of T_3 (Lorijan *et al*, 1980).

Prevention of maturation of the reabsorptive response to adrenaline or cAMP by fetal thyroidectomy.

Thyroidectomy prevented most of the maturation of the responsiveness to adrenaline except

in the one fetus that showed biochemical evidence of thyroid regeneration. The small reabsorptive response seen in 3 of the 17 thyroidectomised fetuses late in gestation could have been due to minimal feto-maternal transfer of thyroid hormones but we were unable to show a relationship between residual thyroid hormone concentrations and responsiveness to adrenaline in the thyroidectomised group. Thyroidectomy also impaired the reabsorptive response to cAMP. In the group of Tx fetuses to which cAMP was added to lung liquid to stimulate absorption there was no evidence of any maturation of the reabsorptive response during the 3 week study period. Since the use of cAMP by-passes the ß-adrenoreceptor it follows that the effect of thyroid hormones cannot be attributed to their action on these receptors. This conclusion is in accord with the observation that thyroid hormones do not affect ß-adrenoreceptor maturation in the fetal lungs of rabbits or rats (Giannopolous and Somers-Smith, 1982; Whitsett *et al*, 1982). It is thus likely that intracellular maturational events distal to the generation of cAMP are affected by thyroid hormones.

Restoration of the reabsorptive response: T_3 and T_4 replacement studies in thyroidectomised fetuses.

In experiments where thyroid hormones were replaced in thyroidectomised fetuses, infusion of T_4 from 118 days at a rate equivalent to mature fetal T_4 production (Nathanielsz *et al*, 1973) leads to maturation of adrenaline responsiveness in the normal way. This confirms the dependence of the reabsorptive mechanism in fetal alveolar epithelium on normal fetal thyroid hormone production during the final weeks of gestation.

A similar pattern of maturation was seen in fetuses infused with the active hormone T_3 from 118 days. Again, lung liquid reabsorption during adrenaline infusion was seen only after 130 days while fetuses infused with T_3 from 110 days showed only a small advance in the timing of adrenaline responsiveness. Our inability to induce an earlier appearance of this response despite exposure to high concentrations of T_3 in relatively immature fetuses suggests that the normal rise in T_3 during the last 3 weeks of gestation cannot be the only factor controlling the appearance of this reabsorptive capacity. The fetuses given the higher rate of T_3 infusion from 125 and 131 days (following thyroidectomy at 118 days) were refractory to adrenaline infusion for up to 10 days from the start of infusion. The delay in the appearance of the reabsorptive response may be explained by the prolonged period of hypothyroidism experienced by these fetuses following thyroidectomy before T_3 replacement was started compared to the fetuses of the 110 and 118 days groups in which T_3 infusion commenced shortly after thyroidectomy.

Our inability to bring forward the gestation at which reabsorption was first seen and the variability of the rise in T_3 seen after 130 days in the normal fetus suggests that T_3 has a permissive effect on development of the reabsorptive mechanism. It is also clear that the plasma concentration of T_3 above a threshold value is not critically important in determining the magnitude of the response since there was no relationship between the relatively high T_3 concentrations seen in the T_3-infused fetuses and reabsorption rates after 130 days.

T_3 and hydrocortisone infusion studies.

Synergism of T_3 and cortisol: Our studies in which T_3 and hydrocortisone were infused alone or in combination show that true synergism exists between these hormones in the induction of the reabsorptive response to adrenaline. Infusion of these hormones together into immature fetuses resulted in changes in the ion transport mechanism of the pulmonary epithelium, enabling it to respond to @@-adrenergic stimulation at a much earlier age than is normally observed. Both these hormones are clearly necessary for this development as neither hormone alone produced any change in the response to adrenaline.

The induction of a reabsorptive response to adrenaline, within a few hours of exposure to T_3 and hydrocortisone, occurred at least 10 days earlier than the gestation at which this response is normally first seen in the fetal sheep (Brown *et al*, 1983). It is clear that, even while the fetal lung is at a relatively immature stage of structural development (Alcorn *et al*, 1977), it is possible to manipulate the alveolar ion transport mechanism with the use of these hormones. The complete reversibility of this effect after withdrawal of T_3 and hydrocortisone, and the re-induction of the response to adrenaline following their reintroduction suggest that T_3 and hydrocortisone, acting together, are responsible for the normal maturation of the reabsorptive response in late gestation (Brown *et al*, 1983).

Time- and dose-dependence of the T_3 + hydrocortisone effect. In experiments where adrenaline was infused continuously, the direction of net lung liquid flow switched from secretion to absorption at about 2 hours after first exposure to T_3 and hydrocortisone and

there was a progressive increase in the magnitude of the reabsorptive response thereafter. The progressive increase in the reabsorptive response was determined by the length of time from first exposure to the hormones but the magnitude of the response was also dependent on the level of plasma cortisol. After 72 hours of T_3 and hydrocortisone infusion the mean reabsorptive response to adrenaline was similar, in these fetuses (at 120 days gestation), to the response seen normally in fetuses between 135 and 140 days gestation (Brown *et al*, 1983). Because of the likelihood of early abortion associated with prolonged hydrocortisone infusion (Liggins *et al*, 1968), we did not attempt to assess the effect of combined T_3 and hydrocortisone exposure for longer than 72 hours.

The speed with which the reabsorptive response to adrenaline occurred (2 - 4 hours) is considerably faster than that reported for T_3 and hydrocortisone induced maturation of the surfactant system in the fetal lung (Ballard *et al*, 1984; Gonzales & Ballard, 1984) where a significant response was seen only after 20 hours of hormone exposure. Rapid effects of T_3 within the time scale of our experiments (2 to 4 hours) are, however, well described, both regarding the rapid cellular penetration of T_3 into nuclei (De Groot & Torresani, 1975), and in its stimulation of specific mRNA such as growth hormone mRNA (Seo *et al*, 1978).

The lack of any increase in the reabsorptive capacity after infusion of T_3 or hydrocortisone alone suggest that these hormones are influencing different intracellular events which, only when acting in concert, allow the reabsorptive response to ß-adrenergic stimulation to occur. This is different from the influence of these hormones on surfactant production in the fetal

lung where a synergistic action is apparently restricted to those effects which each hormone can be shown to stimulate independently (Ballard, 1986). The synergistic effects of T_3 and hydrocortisone on lung liquid transport resemble those on surfactant production in that both systems can be activated or augmented by ß-adrenergic stimulation (Warburton *et al*, 1988).

Whereas the magnitude of the reabsorptive response, in our current experiments, was determined by the length of time after first exposure to T_3 and hydrocortisone, there was no dependence of J_v on the concentration of T_3 in the fetal plasma. Once the T_3 & hydrocortisone infusion commenced, the plasma concentrations of cortisol had a critical bearing on the response to adrenaline, irrespective of the T_3 concentrations.(see fig 5, chapter 6). These findings suggest, firstly, that the effect of T_3 is to induce a rapid change in the ion transport system which persists some hours after disappearance of this hormone from the fetal plasma. The critical dependance of J_v on fetal cortisol concentrations, on the other hand, suggests either the production of a protein with a more rapid turnover time, or that cortisol has a direct action on the ion transport mechanism or its controlling ß-adrenergic system.

Likely sites of action of T_3 and hydrocortisone on the ion transport system.

There is conflicting evidence as to whether thyroid hormones have a direct stimulatory effect on the $Na^+/K+$ ATPase pump (for review see De Groot *et al*, 1986) as often these effects have been shown to be secondary to sodium entry into the cell. Any such action on the

Na^+/K^+ ATPase pump by cortisol would be through a weak mineralocorticoid effect. Increasing $Na^+/K+$ ATPase activity, however, would be expected to increase the rate of active Cl^- transfer and thus the resting secretion rate, whereas a diminution of this rate is normally seen in the last 2 weeks of gestation, at a time when the reabsorptive response is developing (Brown *et al*, 1983). It is more likely that the hormones are influencing the responsiveness of the apical Na^+ transport system which is under adrenergic control. During adrenaline stimulation these reversibly activatable Na^+ channels, which are unique in mammalian ion-transporting epithelia, are presumed to be activated by phosphorylation of protein kinase A, following a rise in the concentrations of intracellular cAMP.

At the level of the Na^+ channel, the augmented response to adrenaline may result from an increase in the number of sodium channels (density), or an increase in the activity of pre-existing channels (conductance or open probability). In the amphibian urinary bladder vasopressin have been shown to increase Na^+ transport by increasing channel density (Li et al, 1982). A similar mechanism is thought to be responsible for cAMP activated Na^+ absorption in the frog colon (Krattenmacher et al, 1988). A rapid increase in channel density may result from insertion of pre-existing channels into the apical membrane (Lewis & de Moura, 1984) or activation of pre-existing channels within the membrane which are normally electrically silent. It is possible that in the case of the fetal alveolar epithelium, T3 and hydrocortisone may stimulate production of such "silent" channels which could be rapidly activated or inserted by ß-adrenergic stimulation. The inhibition of T_3 and hydrocortisone activity by cycloheximide would support a hypothesis that the effects of these hormones are

mediated through synthesis of new proteins (?channels).

Alternatively, T_3 and hydrocortisone may affect individual components of the second messenger system which controls activation of the Na^+ channels in these cells. Studies with exogenously provided dibutyryl cAMP (Olver 1987) indicated that maturation of elements of the second messenger pathway more "distal" to the generation of cAMP must be affected by thyroid and steroid hormones. It is likely that Na^+ channel activation results from phosphorylation events which follow a rise in intracellular cAMP. Whitsett (1983) identified, in Type II cells of fetal rat lung, a number of cytosolic proteins which were phosphorylated by cAMP and protein kinase A. It was found that the activity of phosphorylase A, responsible for glycogenolysis, increased dramatically before birth at a time when endogenous cAMP/protein kinase A levels were declining. This increase in phosphorylation activity of phosphorylase A by cAMP may serve as a model to explain the effects of T_3 and hydrocortisone on possible regulators of the Na^+ channel in the fetal lung.

Clinical implications. Of all the pulmonary adaptations taking place at birth, the most obvious, the least studied, and possibly the most important is the change from the liquid-filled to the air-filled state. Likewise, it seems probable that the importance of inadequate reabsorption or continuing secretion of pulmonary liquid in preterm infants has not been adequately recognized as a cause of neonatal respiratory difficulties. It has been known for some time that T_3 and corticosteroids appear to act in concert to advance structural maturation (Gross *et al*, 1984)) and to promote surfactant synthesis in both organ cultures

of the fetal rat lung and in the intact fetal rat (Hitchcock, 1979). More recently Warburton *et al*, 1988) found that administration of corticosteroids and thyrotropin releasing hormone (TRH) together to the fetal sheep led to maturation of the surfactant system and improved lung mechanics. Both these effects were enhanced when a ß-agonist was infused in addition to the hormones. Similarly Schellenberg *et al* (1988) have shown that a combination of T_3, hydrocortisone and prolactin infused into the fetal sheep increased lung distensibility and stability. Interestingly, least studied, and possibly the most important is the change from the liquid-filled to the air-filled state. Likewise, it seems probable that the importance of inadequate reabsorption or continuing secretion of pulmonary liquid in preterm infants has not been adequately recognized as a cause of neonatal respiratory difficulties. Interestingly, Liggins *et al* (1988) found that while a significant increase in saturated phosphatidylcholine from alveolar lavage occurred in response to TRH infused to sheep fetuses from 124 to 128 days gestation, improvement in lung distensibility and stability was not seen unless TRH was infused together with cortisol. In the light of our present findings, these results may have been partly or mainly due to the effect of these hormones on the pulmonary liquid reabsorptive system.

In the context of the present experiments, very preterm infants are most likely to be born at a stage when the lungs have had insufficient pre-natal exposure to T_3 and corticosteroid hormones. Mean cord concentrations of both these hormones are known to be significantly lower in infants born preterm (Cuestas, 1978; Sybulski *et al*, 1976) and it has been shown that within the preterm group low cord concentrations of both thyroid hormones (Cuestas

et al, 1976) and cortisol (Sybulski *et al*, 1976) at birth are associated with a higher incidence of subsequent respiratory problems. Lucas *et al*(1988) report that 50% of T_3 concentrations measured during the first week of life in infants under 1200 g were below 0.19 ng/ml (0.3nM) whereas our present data suggest that concentrations over 0.3 ng/ml may be required to induce the reabsorptive mechanism in the fetal sheep. Whereas it appears that many pre-term infants are able to mount a substantial post-natal surge in T_3 (Cuestas, 1978), this would be too late to prevent the onset of respiratory difficulties which may be associated with poor lung liquid clearance in the first 24 hours of life.

Our experiments show that, with the exogenous administration of T_3 and hydrocortisone,the reabsorptive response to adrenaline can be induced much earlier in gestation, and within a much shorter time-scale than is normally seen. The relative independence of this aspect of lung development from other aspects of development and its ability to respond to hormones at a very early stage of gestation may be important factors in determining the survival of those very small pre-term infants who have had insufficient pre-natal exposure to T_3 and hydrocortisone. Since cortisol and T_3 concentrations can be manipulated both pre- and post-natally (Roti *et al*, 1981; Ballard & Liggins, 1982), it may be possible use these hormones to enhance the lung liquid reabsorptive capacity of the immature lung.

Future directions.

As was stated earlier, it is likely that the site of adrenaline-sensitive sodium absorption in the fetal lung is at the alveolar, rather than the airways epithelium. Because of the complex architecture of the distal lung, it is not possible to study ion transport across excised preparations of this region as has been done with tissue from conducting airways. Methods derived from similar adult lung studies could be utilised to develop planar preparations of enzymically disaggregated distal fetal lung which could then be subjected to electrophysiological study. Recent advances in genetics and electrophysiology should enable more detailed study of this region of the fetal lung at a cellular and molecular level.

New and established techniques in electrophysiology (Ussing studies, intracellular microelectrode recordings, patch clamping, noise analysis) can be used to confirm which region of the lung the hormone effect is being exerted and to elucidate the underlying changes in ion transport which determine the appearance of the adrenaline sensitive absorptive effect. It should thus be possible to determine whether the priming effect of the hormones result from changes to the Na^+ channels themselves or regulators of the sodium channel, as discussed earlier.

The cloning of genes which code for ion transport pumps (e.g Na^+/K^+ ATPase) and the cystic fibrosis transmembrane regulator (Riordan *et al*, 1989), may provide insight into which is likely to control Cl^- transport, The findings in our study that adrenaline-sensitive Na^+ reabsorption can be manipulated by T_3 and hydrocortisone may provide a useful opportunity

to determine which components of the ion transport system (channels, channels regulators, or pumps) and potential controlling genes (via transcription or translation) may be responsible for maturation of ion transport in the fetal lung.

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CONCLUSION.

This study has shown that fetal thyroid and steroid hormones are critically important in determining the appearance and the magnitude of the reabsorptive response to adrenaline that develops in the lungs of fetal sheep in late gestation. Through the technique of thyroidectomy and subsequent thyroid hormone replacement, we have gathered valuable new information on thyroid hormone kinetics in the fetus as well as on the importance of these hormones in determining ion transport characteristics of the developing fetal lung. Experiments with dibutyryl cAMP implicated an intracellular site for the action of thyroid hormones rather than an effect of these hormones on the ß-receptor itself. Co-infusion experiments with steroid and thyroid hormones provide clear evidence for a synergistic interaction between these 2 hormones in the development of the reabsorptive response. The action of these hormones is rapid and, at the gestations tested, is fully reversible. Whilst there is some evidence for a permissive role for thyroid hormones, the close correlation between cortisol concentrations and the magnitude of the reabsorptive response in the co-infusion experiments indicates a more direct role for cortisol. The observation that the synergistic effect can be prevented by pre-exposure of the lung to cycloheximide suggests that these hormones exert their effect through new protein formation. Finally, these findings may be of importance, not only in areas of fetal development which are poorly understood, but also in the clinical management of pre-term infants with respiratory diseases of prematurity.

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