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EPITHELIAL SOLUTE PERMEABILITY, ION TRANSPORT AND TIGHT JUNCTION MORPHOLOGY IN THE DEVELOPING LUNG OF THE FETAL LAMB

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SUMMARY

1. Experiments were performed on exteriorized fetal lambs of between 69 days' gestation and term (147 days) in order to observe changes in lung volume and lung liquid secretion rate, and to delineate any alterations in solute permeability, ion transport and tight junction morphology in the maturing lung epithelium. Whilst it was technically possible to measure solute permeability as early as 69 days it was not feasible to apply the Ussing flux ratio technique before 84 days.

2. Fetal lung liquid volume and secretion rate, when normalized for body weight, increase linearly with gestation, whereas tracheal volume expressed in the same manner remains constant.

3. When expressed in terms of pore theory, epithelial permeability to small polar non-electrolytes does not change between 69 days and term (equivalent pore radius 0·66 nm and 0·64 nm respectively).

4. In the immature fetus of 69–76 days, mean epithelial tight junction strand number is 8·3, whereas by the end of gestation it has fallen to 4·6.

5. The transfer constants (min^{-1}) for sodium and chloride movement in the direction lung liquid to plasma are, respectively, some 6 and 4 times greater at 84–87 days than at term.

6. As in the mature fetus, the lung epithelium at 84–87 days actively transports chloride from plasma to lung lumen, albeit with a slightly reduced transport e.m.f. Sodium movement does not, at any gestational age, differ from the predictions for passive transfer.

7. In lung liquid the concentrations of chloride and potassium increase and that of bicarbonate decreases during gestation, whilst that of sodium does not change. The rises in lung liquid chloride and potassium concentrations follow those in plasma, maintaining plasma/lung liquid ratios of 0·7 and 0·95 respectively. However, plasma bicarbonate remains constant and the plasma/lung liquid ratio for bicarbonate rises from 3 at 69–76 days to 20 near term as the lung liquid bicarbonate falls from 9·8 to under 2 m-mole kg^{-1} H_2O.

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8. Whereas lung liquid protein concentration remains constant and low at about 0·35 g $l.^{-1}$, plasma protein concentration rises from 23 g $l.^{-1}$ at 69–76 days to 43 g $l.^{-1}$ near term. During the same period arterial blood pressure doubles.

INTRODUCTION

The lumen of the fetal lamb lung is filled with a liquid of unique composition (Adams, Moss & Fagan, 1963; Adamson, Boyd, Platt & Strang, 1969) which plays an important role in lung development (Alcorn, Adamson, Lambert & Maloney, 1977). Near term (147 days' gestation) this liquid is formed at a rate of up to half a litre per day by a process involving active transport of chloride ions by the pulmonary epithelium (Olver & Strang, 1974). An essential prerequisite for net secretion, that the epithelium be of low permeability (particularly to those ions which are actively transported), was demonstrated by Normand, Olver, Reynolds, Strang & Welch (1971) and Olver & Strang (1974) in fetal lambs of between 120 days and term.

As a way of investigating the relationship between lung structure and secretory function we have measured epithelial permeability and secretion early in gestation at a time when lung morphology is strikingly different from that at term.

Preliminary observations in lamb fetuses of about 70 days' gestation showed that lung liquid was present, but that its ionic composition was closer to that of an ultrafiltrate of plasma than was the lung liquid of more mature fetuses. We considered that the secretory system of the lung epithelium might be in some way less efficient in the immature fetus. This would occur if the available force (per unit surface area) for active ion transport were diminished, leading to a relatively smaller one-way flux from plasma to lung lumen, or if epithelial permeability to solutes were increased, leading to a relatively greater passive 'back-flux'.

We examined these possibilities in a series of experiments in which measurements were made which permitted assessment, at an early stage of lung development (69–87 days' gestation), of alveolar permeability to electrolytes and non-electrolytes and which allowed us to calculate transport e.m.f. In addition, we obtained electron micrograph freeze-fracture replicas of alveolar tight junctions at various gestational ages between 69 days and term, with a view to determining whether any changes in epithelial permeability might be related to changes in the number of intramembraneous strands which run parallel to the plane of the epithelium and which, by partially occluding the tight junction, are thought to have a 'sealing' function. The data collated by Claude & Goodenough (1973) from several sources, with observations on several different epithelia, suggest that tight junction strand number is inversely related to electrical conductance and hence, presumably, is inversely related to solute permeability.

METHODS

Experiments were performed on pregnant Dorset Short Horn and Clun Forest ewes, tupped on known dates. Fetal lambs between 69 days' gestation and term (147 days) were exteriorized by hysterotomy under chloralose anaesthesia, as previously described (Normand *et al.* 1971). On delivery from the uterus, fetuses less than about 100 days were completely covered in transparent cling-film to prevent drying of the hairless skin. All fetuses were delivered on to a heated table and covered with wool, body temperature being continuously monitored by means of a rectal thermistor

and kept between 38 and 40 °C. A tracheal cannula was inserted within minutes of delivery and catheters were placed in a jugular vein and carotid artery. Arterial blood pressure and heart rate were monitored continuously, the pressure transducer being placed level with the lamb's mid-thorax. Arterial P_{O_2} P_{CO_2} and pH were measured intermittently during the experiment. Any lamb which had a pH less than 7·20 at the beginning of the experiment was not used for studies of lung liquid secretion or composition.

Injection of tracers and sampling of fluids

The procedures followed were closely similar to those described by Normand *et al.* (1971) and Olver & Strang (1974). After an interval of 30 min from the time of injection of tracers into the circulation or lung liquid, samples of blood (0·5–1·5 ml.) and lung liquid (0·1–0·5 ml.) were taken, the precise rate of sampling of lung liquid being adjusted to equal its secretion rate in an attempt to keep lung liquid volume constant.

Lung liquid and plasma composition

Where possible, paired lung liquid and blood samples were taken shortly after delivery for chemical analysis. Blood samples were centrifuged and the plasma separated within 2 hr; all samples were deep-frozen until analysed. For details of chemical analyses see Adamson *et al.* (1969) and Olver & Strang (1974). In calculating chemical activity of ions in plasma the following equation was used: $W = 984 - 7{\cdot}18P$, where W is water concentration (ml. $l.^{-1}$) and P is total plasma protein concentration (g $l.^{-1}$) (Eisenman, Mackenzie & Peters, 1936). Lung liquid water content was determined in fifteen samples by evaporating to dryness and was found to be $991 \pm 1{\cdot}5$ ml. H_2O $l.^{-1}$ of lung liquid (gestation range 77–106 days, mean 91·3 days). There was no correlation with gestational age.

Lung liquid volume and secretion rate

Lung liquid secretion rate was measured by the impermeant tracer technique, ^{131}I- or ^{125}I-labelled albumin (Radiochemicals, Amersham) being used (Olver & Strang, 1974). In preliminary experiments, performed to validate the use of this tracer in immature fetuses, radioiodine-labelled albumin and [^{14}C]inulin were injected I.V. and lung liquid samples and blood samples taken (see above). In all cases very small quantities of both tracers were found in lung liquid and their concentrations increased with time. Lung liquid/plasma tracer concentration ratios (LL/P) were calculated from the samples taken 100 min after injection. For [^{14}C]inulin these were (mean ± S.E.M.):

70–79 days	LL/P = 0·0151 ± 0·0064	$n = 6$
80–89 days	LL/P = 0·0098 ± 0·0022	$n = 7$
90–113 days	LL/P = 0·0031 ± 0·0021	$n = 7$

In four of these experiments no mixing of the lung liquid was carried out until a sample was taken 100 min after I.V. injection. The LL/P ratios were no less in these experiments than those in which regular mixing was performed, indicating that mixing did not damage the epithelium in a way which increased its permeability. For radiolabelled albumin the mean LL/P ratio at 100 min was:

73–111 days	LL/P = 0·0043 ± 0·0011 (range 0·001–0·0071)	$n = 5$

These figures indicate that the pulmonary epithelium has a low permeability early in gestation and that the penetration by radiolabelled albumin over the usual experimental period is so small as to allow it to be used as a lung liquid volume marker. When using radioiodine-labelled albumin in this way it was first mixed with lung liquid taken from the experimental fetus and equilibrated over an ion-exchange resin (Iobeads) for 7–10 min before being injected into the lung via the trachea. The volume of lung liquid at each sample time and the secretion rate were calculated as shown below. Four to nine ponts were used to calculate the regression line of volume against time. The correlation coefficient was greater than 0·90 in forty-four (92 %) experiments but in five others the points showed considerable scatter and the slopes of the regression lines were not significantly different from zero; these experiments were used in the calculation of lung liquid volume but the estimates of secretion rate are not included in Table 1.

'Tracheal' volume

At the end of the experiment, after the fetus had been killed, the circumference of the trachea and its length between the tracheotomy and the carena were measured. It was assumed that the shape of the trachea approximated to a cylinder and its volume calculated on this basis. In the small fetuses the trachea was circular in cross-section, but near term the cross-section was pear-shaped, with the 'point' lying posteriorly. Thus in the mature fetuses the calculated volume is likely to be a small over-estimate.

Permeability experiments

Permeability of the pulmonary epithelium was measured from the lung lumen side in eight fetuses between 69 and 76 days' gestation. These were the smallest fetuses on which our techniques could be applied with confidence. [^{131}I]albumin, as a volume marker, and two non-electrolyte water-soluble radiolabelled probes (0·4–2·5 μc) of a possible four ([^{3}H]sucrose, [^{3}H]mannitol, [^{14}C]erythritol, [^{14}C]urea) were mixed into the lung liquid at zero time. Approximate volume of tracer plus carrier added was 35 μl. Calculated changes in lung liquid volume attributable to the osmotic effect of adding the carriers were betwen -10 and $+9$ μl. At the end of the measurement periods the mean plasma/lung liquid concentration ratios of the probes were: sucrose 0·1 %, mannitol 0·04 %, erythritol 0·2 % and urea 1·2 %. In one experiment, where ^{14}C was the label on both the non-electrolyte probes, separation was achieved by Sephadex gel filtration (G15) before scintillation counting. The purities of the stock solutions of the non-electrolyte probes were checked by Sephadex gel filtration before and after the series of experiments.

Ion flux and electrical potential difference experiments

Lambs of about 84–87 days' gestation were used for these experiments, since they were the smallest fetuses in which sufficient lung liquid could be sampled for chemical analysis and still leave enough for an experiment to be performed. ^{24}Na and ^{36}Cl fluxes were measured after their introduction into lung liquid and [^{125}I]albumin was used as the volume marker. Approximate volume of tracer (0·4–10 μc) plus carrier added was 100 μl. Calculated changes in lung liquid volume due to osmotic effects of the carriers were between $+3·3$ and -17 μl. Because sodium passes very rapidly out of lung liquid into the circulation, paired lung liquid and blood samples were always taken. The mean plasma ^{24}Na concentration was 61·1 % (S.E. $\pm$10·1 %, $n = 6$) of the lung liquid concentration at the end of the measurement periods. Hence only early data points were used in the calculation of K_0 for sodium (see Fig. 3). Chloride crosses the pulmonary epithelium much more slowly and the mean plasma concentration at the end of the experiments was only 1·2 % ($\pm$0·3 %) of lung liquid concentration. In four fetuses electrical potential difference between lung lumen and blood was measured with a Vibron electometer (input impedance 10^{14} Ω) connected via calomel half-cells to potassium chloride–agar bridges (3 M-potassium chloride in 4 % agar; tube diameter: inside 0·64 mm, outside 1·4 mm). One of the bridges was inserted down a side arm of the endotracheal tube through a self-sealing rubber cap until it met resistance, when it was withdrawn 1–2 cm; the other was inserted into the jugular vein for a distance of 3–4 cm. After all lung liquid samples had been taken, the calomel electrodes were re-checked for asymmetry potentials and the positions of the agar bridges in lung and blood reversed. At the end of the experiment the lung was dissected, examined for evidence of damage and the position of the lung bridge noted.

Isotope counting

The weighed samples of lung liquid and plasma were counted on a Packard Autogamma Scintillation Spectrometer (Model 3003) and a Packard Tricarb. Liquid Scintillation Spectrometer (Model 3375).

Blood-gas analysis, heart rate and blood pressure

Blood-gas analysis were performed at least once on every fetus, using a Radiometer blood-gas analyser. The initial measurements (mean $\pm$ S.E.M.) in the ninety-seven fetuses used in the study were: pH 7·35 ($\pm$0·006, range 7·23–7·50); arterial P_{O_2}, 27·3 ($\pm$0·6, range 14–42 mmHg); arterial P_{CO_2}, 54·7 ($\pm$0·9, range 35–70 mmHg); heart-rate, 189·4 ($\pm$4·2, range 120–280). Blood pressure increased with gestational age (see Results). Using a paired t test to compare initial and final measurements, the only value to show a significant change during the experimental period was mean

arterial blood pressure, which decreased by an average of 4 mmHg in the eight fetuses used for non-electrolyte permeability measurements.

Fixation and morphology

Morphological studies were carried out in a group of immature fetuses correspondong to those in which measurements of non-electrolyte permeability were made (69–76 days' gestation) and in three mature fetal lambs (140–142 days). Fixation was achieved by aspirating as much lung liquid as could be removed and replacing it with a similar volume of a solution containing 1·3% formaldehyde and 1·6% glutaraldehyde in 0·1 M-cacodylate buffer at pH 7·3. Small pieces of lung were later fractured at −150 °C in a Balzer's high vacuum freeze-etch unit and carbon–platinum replicas prepared. (For full details see Schneeberger, Walters & Olver, 1978). High-magnification photographs (× 50,000 to × 82,500) were taken and strand counts performed independently by the two authors who were unaware of the gestation of the fetuses from which the tissue was taken. A grid of 0·25 μm spacing was placed on the micrographs so that the grid lines lay perpendicular to the longitudinal axis of the tight junction network. The number of strands intersected by each grid line and the total length of junction examined were counted. Care was taken to count only those portions of the junctions which appeared complete in the fracture plane of the replica. In all, 62 μm of tight junction were examined in seven immature fetuses.

Calculations

Finding lung liquid volume V_L and secretion rate (J_v). At time t,

$$V_t = [(X-R)/C_t] + V_s, \tag{1}$$

where V_t is accumulated lung liquid volume, X is the amount of impermeant tracer added, R is the sum of the amounts of tracer removed in previous samples, C_t is the concentration of tracer in the sample taken at time t, and V_s is the sum of the volumes of all previous samples.

The slope of the regression of V_t against t gives the secretion rate J_v and the intercept on the ordinate gives V_L, the volume of liquid present in the lung at zero time.

Finding γ and K_0 (see Olver & Strang, 1974). Provided that the concentration of tracer in plasma remains low, and hence back-flux can be ignored, the concentration of tracer in lung liquid (C_L) can be expressed as a simple exponential function of time (t):

$$\ln C_L = \ln C_L^0 - (K_0+\gamma)t, \tag{2}$$

where C_L^0 is concentration in lung liquid at zero time K_0 is the constant (min^{-1}) for transfer out of lung liquid across the pulmonary epithelium, and γ is J_v/V_L (min^{-1}).

Ion flux and transport e.m.f. (see Olver & Strang, 1974). For any ion, the ratio of the one-way fluxes across the lung epithelium is given by:

$$\frac{J_{PL}}{J_{LP}} = \frac{K_0+\gamma}{K_0}, \tag{3}$$

where J_{PL} is the one-way flux from plasma to lung liquid and J_{LP} is the one-way flux in the reverse direction.

The flux ratios for sodium and chloride can thus be calculated from the experimental values given in Table 5 and can be compared with the flux ratios predicted for passive transfer according to the flux-ratio equation of Koefoed-Johnson & Ussing (1953). Subtraction of the predicted flux ratio (for *passive* movement of ions) from the observed flux ratio gives Δ which, if expressed in millivolts, is a measure of the 'transport e.m.f.'. Transport e.m.f. = $\Delta RT/ZF = \Delta 26{\cdot}8$ (see Olver & Strang, 1974, p. 343, and Ussing & Zerhan, 1951).

Finding pore radius, r (see Normand *et al.* 1971, p. 321). According to pore theory (Landis & Pappenheimer, 1963; Solomon, 1968), where transfer is determined by restricted diffusion, the values of K_0 in eqn. (2) and (3) are given by:

$$K_0 = [(A/dx)/V] \,.\, D_t \,.\, F(a/r), \tag{4}$$

A/dx is the pore area per unit path length, V is the volume of lung fluid, D_t is the free diffusion coefficient at body temperature t, and $F(a/r)$ is a function of (a/r), where a is molecular radius and r is the pore radius. $F(a/r)$ is usually given (see Renkin, 1954) as:

$$F(a/r) = [(1-a/r)^2][1-2{\cdot}104(a/r)+2{\cdot}09(a/r)^3-0{\cdot}95(a/r)^5], \tag{5}$$

K_0, D_t and a being known, the value of r which gave the minimum variance for the set of 'area' terms, $(A/dx)/V$, corresponding to a set of molecular probes, was taken as the best fit value of r. The value so calculated for each fetus was very similar to, and sometimes identical with, that found by a least squares fit. The former method was used since it gives equal weight to all the points. Normand *et al.* (1971), using an iterative least squares technique, estimated the pore radius to be 0·55 nm for the mature fetus. Reworking their data using the minimum variance method gives a value of 0·64 nm.

TABLE 1. Some physical measurements performed on fetal lambs grouped in 10-day gestation periods. Mean values followed by S.E.M.s are given, with number of fetuses in brackets. V_L is lung liquid volume, J_v lung liquid secretion rate, V_t 'tracheal' volume and A_t 'tracheal' surface area

Gestation (days)	Bodyweight (kg)	Wet lung tissue weight (g)	Dry lung weight (g)	V_L (ml.)	J_v (ml. hr^{-1})	V_t (ml.)	A_t (cm^2)
74·1	0·231	11·31	1·055	0·955	0·379	0·295	4·34
0·68	0·017	0·59	0·119	0·220	0·039	0·027	0·30
(16)	(16)	(13)	(7)	(11)	(10)	(10)	(10)
84·8	0·479	21·62	2·690	2·993	1·003	0·831	9·00
0·55	0·024	1·05	0·272	0·368	0·075	0·079	0·60
(21)	(20)	(15)	(4)	(19)	(19)	(7)	(7)
96·1	0·840	33·116	—	9·298	1·504	1·603	13·03
0·93	0·033	1·959	—	1·937	0·203	0·170	0·76
(9)	(9)	(5)		(5)	(4)	(4)	(4)
105·2	1·263	42·58	9·04	34·57	3·252	3·85	24·2
1·6	0·216	10·28	—	4·92	1·242	—	—
(5)	(5)	(4)	(1)	(4)	(4)	(1)	(1)
113·4	2·031	56·70	9·643	49·17	4·16	3·69	22·62
0·70	0·076	—	0·811	3·42	0·53	—	
(12)	(12)	(1)	(6)	(4)	(3)	(2)	(2)
125·31	2·931	69·1	11·732	69·52	10·77	5·07	28·13
0·72	0·172	—	0·920	7·09	1·54	—	—
(13)	(12)	(1)	(7)	(6)	(6)	(2)	(2)
134·9	3·42	51·95	17·353	85·41	10·04	5·37	28·49
1·03	0·23	—	1·881	16·47	4·04	0·64	2·76
(15)	(15)	(2)	(6)	(4)	(3)	(5)	(5)
126·4*	2·809	73·09	—	76·23	7·25	—	—
0·42	0·128	6·52	—	4·99	0·89	—	—
(22)	(19)	(12)		(22)	(13)		
140·0*	3·815	64·95	—	122·6	9·20	—	—
0·19	0·093	2·92	—	3·75	0·56	—	—
(74)	(71)	(46)		(73)	(42)	—	

* Data for these gestations are from original records of experiments reported by Olver & Strang (1974).

RESULTS

Lung liquid volume (V_L) and tracheal volume

The volume of liquid (V_L) contained within the lungs of fetal lambs, normalized for body weight, increases with gestation (Table 1). Parameters of the regression line are: slope (ml. kg^{-1} day^{-1}) = 0·413 and ordinate intercept (ml. kg^{-1}) = −27·72;

$r = 0{\cdot}86$, $n = 51$ and $P < 0{\cdot}001$. In contrast, tracheal volume per unit body weight remains constant (mean value = $1{\cdot}57 \pm 0{\cdot}057$ ml. kg^{-1}; $n = 31$).

Lung liquid secretion rate (J_v)

Lung liquid secretion rate (J_v) increases with gestation (Table 1). The relationship between J_v, expressed per gram of wet lung tissue weight, and gestation (69–142 days) is linear. Parameters of the regression line are: slope (ml. hr^{-1} g^{-1} day^{-1}) = 0·0023 and ordinate intercept (ml. hr^{-1} g^{-1}) = −0·1413; $r = 0{\cdot}87$, $n = 34$ and $P < 0{\cdot}001$. 'Wet lung weight' is the weight of the lung excluding lung liquid. If the lung is evaporated to constant weight the relationship between J_v, per gram dry weight, and gestation (69–121 days) is also linear: slope (ml. hr^{-1} g^{-1} day^{-1}) = 0·0138, ordinate intercept (ml. hr^{-1} g^{-1}) = −0·7584, $r = 0{\cdot}84$, $n = 12$ and $P < 0{\cdot}001$.

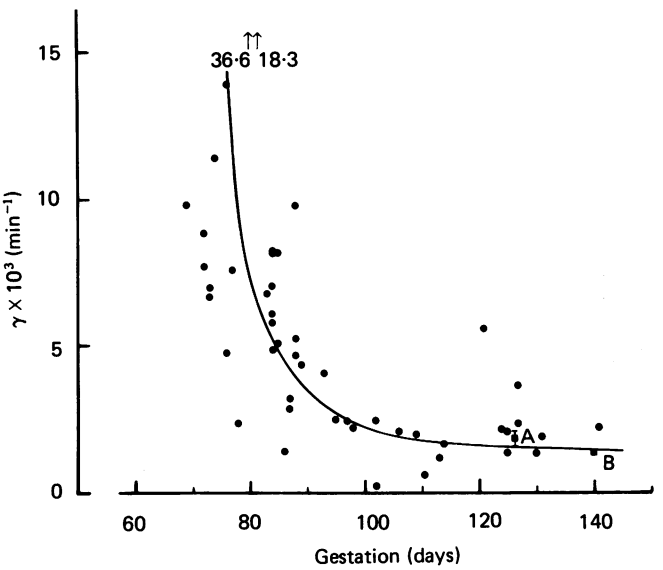

Fig. 1. Change in γ ($= J_v / V_L$) with gestation in fetal lambs. Each point (filled circles) is obtained from one fetus. The positions of two values of γ (36·6 and $18{\cdot}3 \times 10^3$ min^{-1}), which lie outside the ordinate scale, are indicated by the arrows. The line is drawn by eye. Points A and B (filled squares) represent data from Olver & Strang (1974). Point A: mean $\gamma = 1{\cdot}81 \times 10^3 \pm 0{\cdot}25 \times 10^3$ min^{-1}; $n = 13$; gestation range 122–129 days, mean gestation $126{\cdot}3 \pm 0{\cdot}6$ days. Point B: mean $\gamma = 1{\cdot}35 \times 10^3 \pm 0{\cdot}09 \times 10^3$ min^{-1}; $n = 42$; gestation range 137–143 days, mean gestation $139{\cdot}9 \pm 0{\cdot}3$ days.

No dry lung weight data are available from Olver & Strang's (1974) experiments for comparison. The time taken to replace completely a lung volume with newly formed lung liquid (the turnover time) is between 1 and 4 hr at 70–90 days' gestation whilst, near term, this turnover time is 12 hr or more (the corresponding values for γ ($= J_v / V_L$) are 0·017 and 0·004 min^{-1}: see Fig. 1).

Chemical composition of lung liquid

Table 2 gives the mean values for concentration of the main chemical constituents of lung liquid and plasma between 70 days' gestation and term. Table 3 expresses

TABLE 2. Mean values of the main constituents of fetal lung liquid (LL) and plasma (P) between 70 days' gestation and term in 10-day gestation periods. Mean and S.E.M. are given, with number of fetuses in brackets

Gestation (days)	Sodium		Potassium		Chloride		Bicarbonate		Protein	
	LL	P	LL	P	LL	P	LL	P	LL	P
74·2	149·9	144·4	4·62	3·68	144·8	104·02	9·79	26·72	0·281	22·80
0·89	1·73	1·11	0·17	0·40	1·91	1·59	0·84	1·71	0·027	1·78
(12)	(5)	(6)	(4)	(6)	(8)	(6)	(11)	(6)	(3)	(6)
85·1	148·7	147·8	4·30	3·96	142·9	102·75	6·82	25·25	0·341	25·39
0·64	1·61	2·43	0·09	0·07	1·50	1·52	0·57	1·88	0·038	0·93
(18)	(17)	(11)	(17)	(11)	(17)	(11)	(14)	(10)	(5)	(11)
96·2	142·9	144·3	4·02	3·68	141·2	101·1	3·80	24·8	0·378	27·80
0·83	1·04	1·03	0·16	0·19	0·65	0·6	0·56	2·09	0·032	0·72
(10)	(10)	(8)	(9)	(8)	(10)	(8)	(10)	(6)	(9)	(8)
105·2	144·9	149·4	4·37	3·78	141·4	102·4	6·39	30·8	0·485	30·34
1·56	3·64	0·44	0·32	0·24	3·02	0·82	1·49	2·18		1·73
(5)	(4)	(5)	(4)	(5)	(4)	(5)	(4)	(4)	(1)	(5)
112·7	141·7	146·4	4·90	4·96	147·9	105·6	3·44	25·3	0·496	33·48
0·45	2·17	2·54	0·36	0·54	3·66	0·52	0·52	2·16	0·046	1·29
(7)	(7)	(5)	(7)	(5)	(7)	(5)	(7)	(5)	(7)	(5)
125·0	141·9	149·4	4·65	4·5	149·8	102·9	3·20	29·1	0·226	34·70
0·78					3·71		0·46		0·059	
(7)	(2)	(2)	(2)	(2)	(7)	(2)	(6)	(1)	(3)	(2)
137·1	157·1	157·2	5·06	4·78	153·3	111·5	1·80	23·5	0·345	42·68
0·87	4·24	2·74	0·50	0·17	1·29	3·64	0·19	3·03	0·056	1·44
(14)	(6)	(4)	(6)	(4)	(14)	(4)	(13)	(3)	(8)	(4)

All values are in m-mole kg^{-1} H_2O, except for protein values, which are in g $l.^{-1}$.

the same data as the parameters of the linear regression lines (concentration against gestation) calculated for each constituent.

Lung liquid bicarbonate concentration decreases with advancing gestation while plasma bicarbonate does not alter significantly. Consequently the ratio (plasma bicarbonate)/(lung liquid bicarbonate) increases from about 3 at 70 days to about 20 near term. This change in gradient across the lung epithelium is very much greater than for the other ions. (It is curious that the regression line for lung liquid bicarbonate cuts the gestation axis at 148·9 days, which is close to full term in the sheep.)

TABLE 3. Values for the parameters of the regression lines calculated for the main chemical constituents of fetal lung liquid (LL) and plasma (P). Concentration against gestation (in days)

Constituent	Slope + S.E.*	*P* of slope	*r*	Ordinate intercept†	*n*
Sodium					
in LL	0·0	> 0·40	0·124	147·11‡	51
in P	0·1457 ± 0·0439	< 0·005	0·470	133·26	41
Potassium					
in LL	0·0127 ± 0·0050	< 0·02	0·345	3·19	49
in P	0·0177 ± 0·0054	< 0·005	0·463	2·33	41
Chloride					
in LL	0·1716 ± 0·0337	< 0·001	0·534	128·35	67
in P	0·1051 ± 0·0352	< 0·005	0·431	93·43	41
Bicarbonate					
in LL	−0·1124 ± 0·0113	≪ 0·001	−0·783	16·74	65
in P	0·0	> 0·60	−0·091	26·03‡	35
Protein					
in LL	0·0	> 0·99	0·001	0·37‡	36
in P	0·2990 ± 0·0230	≪ 0·001	0·905	3·02	41

Gestation range is 72–142 days in all cases except for bicarbonate, which is 70–142 days.

* Units m-mole kg^{-1} H_2O day^{-1} except for protein which is given as g kg^{-1} H_2O day^{-1}.

† Units m-mole kg^{-1} H_2O except for protein which is given as g kg^{-1} H_2O.

‡ Where the slope of the regression line is not significantly different from zero the value given for intercept is the mean concentration over the gestation period studied.

Lung liquid chloride concentration increases from 141 mM at 70 days to 154 mM at term (Table 3). Plasma chloride concentration also increases significantly but by rather less, from 101 to 109 mM (Table 3). As a result, the ratio of plasma chloride concentration to that of lung liquid is the same at term as it is at 70 days, *viz.* 0·7.

As gestation proceeds, potassium concentration increases significantly in both lung liquid and plasma but only by about 1 m-mole kg^{-1} H_2O in each case. In thirty-four paired samples of lung and plasma, the potassium concentration was significantly higher in lung liquid than in plasma by an average of 0·25 m-mole kg^{-1} H_2O ($t = 2{\cdot}544$, using a paired t test; $P < 0{\cdot}020$; gestation range 72–142 days). However, no significant change during gestation in the plasma/lung liquid concentration ratio for potassium could be detected (mean value 0·945 ± 0·020).

In contrast to these significant changes in chloride, bicarbonate and potassium concentrations, sodium concentration in lung liquid does not alter between 70 and 142 days' gestation. Its plasma/lung liquid concentration ratio is near unity at all times, ranging from 0·99 at 70 days to 1·03 at 142 days.

Protein concentration and blood pressure

Plasma protein concentration almost doubles from 23 g $l.^{-1}$ at 69–76 days to 43 g $l.^{-1}$ near term, in contrast to the very low and constant protein concentration in lung liquid (Table 2).

Mean arterial blood pressure of the fetuses increased with gestational age in a manner similar to that described by Barcroft (1946). The slope of the regression of blood pressure against gestation is 0·3734 mmHg day^{-1}, with an ordinate intercept of −0·5536 mmHg, $r = 0{\cdot}827$, $n = 97$; P of the slope $\ll 0{\cdot}001$. A good correlation was found between mean arterial pressure and plasma protein concentration: slope = 0·452 g $l.^{-1}$ $mmHg^{-1}$, ordinate intercept = 13·54 g $l.^{-1}$, $r = 0{\cdot}858$, $n = 40$, $P \ll 0{\cdot}001$. Since arterial pressure is a potentially important determinant of capillary filtration pressure it seems appropriate, from a teleological standpoint, that it should be linked to the opposing osmotic force provided by the plasma proteins.

TABLE 4. Results of non-electrolyte permeability experiments

Gestation (days)	$\gamma \times 10^4$ (min^{-1})	$K_0 \times 10^4$ (min^{-1})		n	Pore radius (nm)
69–76 (mean 73·3)	68·59 ± 7·34	15·87 (M) ± 2·14	255·6 (U) ± 22·1	6	0·66 ± 0·03
		65·63 (E)	338·6 (U)	1	
		2·24 (S)	21·0 (E)	1	
123–143*	12·0†	2·07 (S)	6·6 (M)	35	0·64
		27·11 (E)	243·3 (U)		

Pore radius (r) is the value which best fits the function of the ratio of the K_0 values of the non-electrolyte molecular probes (see Methods). Where appropriate the data are given as mean ± S.E.M.

In each animal a pair of permeant tracers was studied simultaneously. S is sucrose (molecular radius, $a = 0{\cdot}51$ nm), M is mannitol ($a = 0{\cdot}42$ nm), E is erythritol ($a = 0{\cdot}35$ nm) and U is urea ($a = 0{\cdot}22$ nm).

* Data from Normand *et al.* (1971).

† Derived from mean values of $J_v + V_L$.

Permeability of the lung epithelium to non-electrolytes

Since it has previously been established that the lung epithelium of the mature fetus (123–143 days) has a very low permeability to small non-electrolytes (Normand *et al.* 1971) we have restricted measurement of epithelial permeability in this study to immature fetuses of between 69 and 76 days' gestation. A typical result of permeability measurement, with tracers placed in lung liquid, is shown in Fig. 2, in which it can be seen that the immature lung epithelium exhibits selective permeability, severely restricting the passage of [^{3}H]mannitol compared with [^{14}C]urea. Furthermore, these values of K_0 differ by a factor of 13·7, whereas the difference in free diffusion coefficients is only twofold. Application of pore theory makes it possible to characterize the epithelium in terms of an ideal membrane penetrated by cylindrical channels (pores) of uniform dimension. Thus the immature lung epithelium (69–76 days) has

a mean equivalent pore radius of 0·66 nm (Table 4), which is very similar to the value recalculated from the data of Normand *et al.* (1971) for the mature fetus; in both cases pore radius has been calculated by 'best fit' (see Methods).

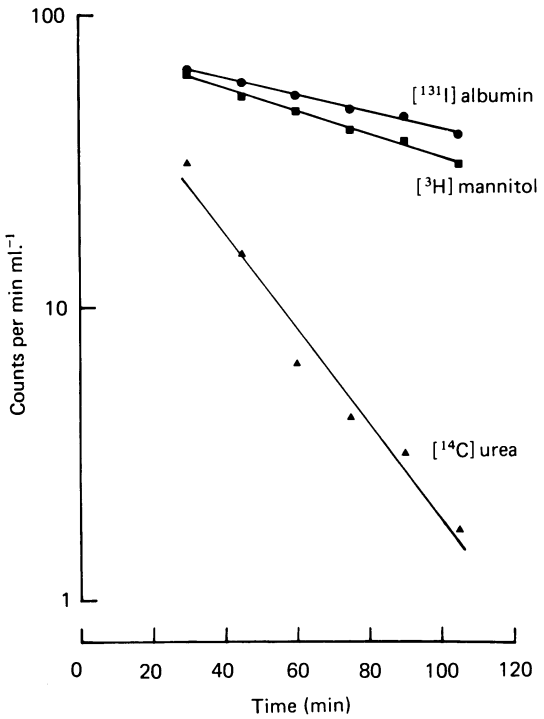

Fig. 2. An example of the non-electrolyte permeability results. In this fetus [^{131}I]albumin, [^{3}H]mannitol and [^{14}C]urea have been introduced into the fetal lung liquid at zero time. Concentration is plotted vertically on a log scale adjusted to juxtapose the calculated regression lines. The slope of the regression lines for each permeant tracer ([^{3}H]mannitol and [^{14}C]urea) equals $K_0+\gamma$. The slope of [^{131}I]albumin, the impermeant tracer, equals γ (see eqn. (2) in Methods). Values for r were all greater than 0·99.

Ion flux, electrical potential difference and transport e.m.f.

Simultaneous measurements of sodium and chloride fluxes were made in six fetuses of 84–87 (mean 84·8) days' gestation, these being the least mature animals in which it was technically feasible to make such observations. The results are set out in Table 5 and an example is shown in Fig. 3. Satisfactory measurements of potential difference were made in four animals (see Methods).

Since there is net secretion of lung liquid in the direction plasma to lung lumen, the flux ratio of each ion (J_{PL}/J_{LP}) is greater than unity. In the case of chloride it is much greater than unity (1·42), reflecting the much lower chloride concentration in plasma compared with lung liquid: the plasma/lung liquid activity ratio (a_P/a_L) for chloride is 0·73. The mean potential difference across the lung epithelium was

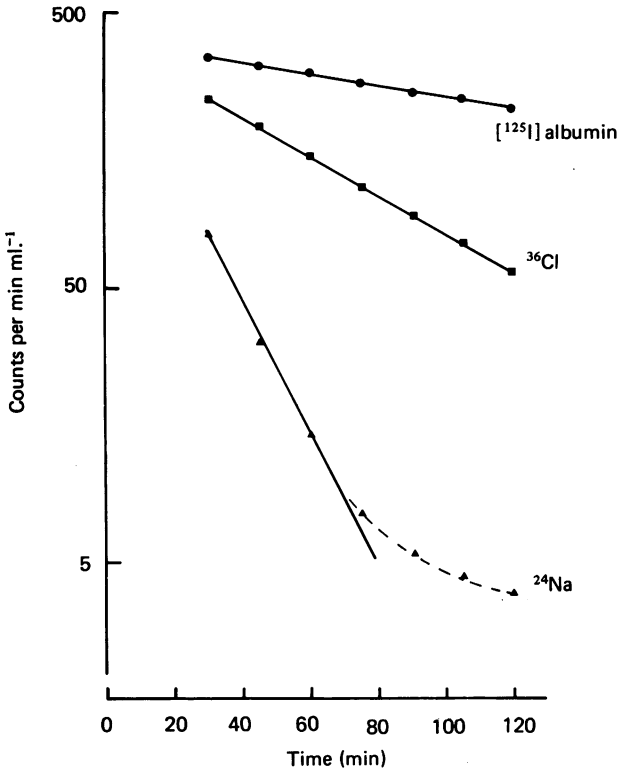

Fig. 3. An example of the results of the ion flux experiments. In this fetus the three tracers, [^{125}I]albumin, ^{36}Cl and ^{24}Na, have been introduced into the lung liquid at zero time. Concentration in lung liquid is plotted vertically on a log scale adjusted to juxtapose the calculated regression lines. The slope of the regression lines of each permeant tracer (^{36}Cl, ^{24}Na) equals $K_0+\gamma$. The slope of the regression line for [^{125}I]albumin (the impermeant tracer) equals γ. Because of the rapid rise in plasma sodium concentration, and consequent back-flux into lung liquid, only the initial linear portion of the slope of log concentration against time has been used in the calculation of K_0 for sodium as in eqn. (2) in Methods. (All values of r were greater than 0·99.)

TABLE 5. Results of electrolyte transport experiments

Ion	K_0 (min^{-1} × 10^3)	a_P (m-mole kg^{-1} H_2O)	a_L (m-mole kg^{-1} H_2O)	J_{PL}/J_{LP}*	Transport e.m.f.* (mV)
Sodium	56·09	144·1	144·9	1·08	−1·1
	±3·24	±3·1	±3·3	±1·02	±0·8
	(9·03)	(154·5)	(150·0)	(1·15)	(−1·5)
Chloride	10·54	101·0	139·5	1·42	+20·5
	±1·12	±2·5	±2·7	±1·04	±1·3
	(2·49)	(102·0)	(150·0)	(1·59)	(+25·8)

In each animal sodium and chloride were studied simultaneously; $n = 6$. Mean values (±S.E.M.) given for K_0, chemical activities in plasma (a_P) and lung liquid (a_L), ratio of one way fluxes, J_{PL}/J_{LP} (where $J_{PL}/J_{LP} = (K_0+\gamma)/K_0$), and transport e.m.f. Mean gestational age = 84·8±1·3 days, mean potential difference = −2·5±0·2 mV, mean value of $\gamma = 4{\cdot}62 \times 10^{-3} \pm 0{\cdot}88 \times 10^{-3}$ min^{-1}.

* Mean of values calculated for each individual experiment.

Figures in parentheses are taken from Olver & Strang (1974), in which gestational age was 123–144 days, $n = 17$.

2·53 mV, lung lumen negative. When the measured flux ratios for sodium and chloride are compared with those predicted for passive transfer (see calculations in Methods) and the difference expressed in units of voltage (transport e.m.f.) we find that, as in the mature fetus, transport e.m.f. for sodium is not significantly different from zero (−1·1 mV) whilst that for chloride is (+20·5 mV). From this we infer that chloride is 'actively' transported. Sodium movement does not differ from the predictions for passive transfer. Table 5 also shows that transport e.m.f., which is a measure of the force acting on an ion causing it to be transported against an electrochemical gradient, is somewhat lower in the immature fetuses studied here than that reported by Olver & Strang (1974) for fetuses closer to term (see Discussion).

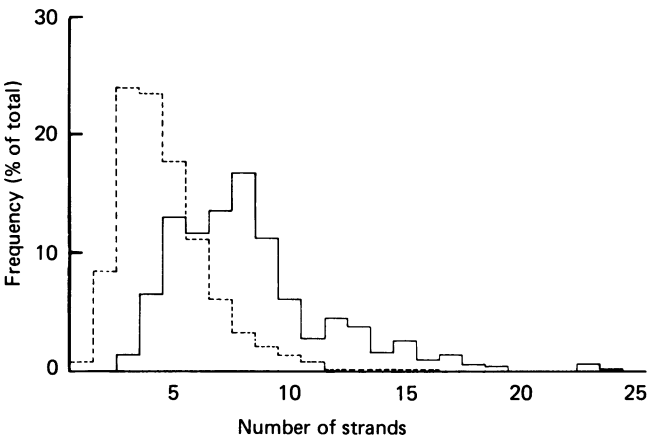

Fig. 4. Distribution of frequencies of the number of strands encountered at 0·25 μm intervals along freeze-fractured tight junctions of the pulmonary epithelium in mature (broken line) and immature (continuous line) lamb fetuses.

Morphological studies

The strand counts performed by the two observers on micrographs of freeze-fractured epithelial tight junctions were in good agreement and hence the mean of the separate counts was used. Histograms of the frequency distribution of the strand counts are shown in Fig. 4 and examples of tight junction replicas are shown in Plate 1. In the seven immature fetuses (69–76 days' gestation) 62 μm of tight junction were examined and 247 counts of strand number made at 0·25 μm intervals; mean number = 8·32 ± 0·16 S.E.M.

In the mature fetuses the junctions between type I alveolar cells (A junctions) and between type I and type II cells (B junctions) were distinguished by one of the authors (E.S.) and the data were analysed separately for each sort of junction. However, no difference in strand number was found between them and therefore the data from A and B junctions were pooled. In these three mature fetuses (140–142 days' gestation) 178 μm of tight junction were examined and 716 counts of strand number made at 0·25 μm intervals; mean strand number = 4·58 ± 0·04 S.E.M. This value differs significantly from that found in the immature group ($P \ll 0{\cdot}001$).

DISCUSSION

Lung volumes and liquid secretion rate

We have shown that lung liquid volume (V_L) increases with advancing gestation while the tracheal volume remains remarkably constant when both are normalized for body weight. The regression line for lung liquid volume cuts the average tracheal volume line at about 70 days, which must mean that almost all the lung liquid at this gestation is contained within the trachea. This is in accord with what is known of fetal lung development in that growth is centrifugal and increasing surface area and volume arise from budding and branching of the bronchial tree distal to the carena. It also suggests that the fetal trachea (or at least the immature fetal trachea) is capable of liquid secretion and that at 70 days it may be the site of most of the secretion. If this is true it should be possible to obtain a rough estimate of water flow per unit area from our measurements of mean total net secretion (J_v) and tracheal area (A_t), *viz.* at 70–80 days' gestation $J_v/A_t = 0{\cdot}379/4{\cdot}34 = 87$ μl. hr^{-1} cm^{-2}, a figure which if high is, nonetheless, of the same order of magnitude as found in certain other tissues (see House, 1974, pp. 428, 429).

In the absence of precise morphometric data we can only make similar calculations for the lung of the mature fetal lamb if we accept that the estimate of 2·8 m^2 for total lung surface (A_L) of the newborn human lung (Dunhill, 1962) is appropriate for the fetal lamb near term. In the chronically catheterized mature fetal lamb, total net secretion (J_v) averages 15 ml. hr^{-1} (Walters & Olver, 1978). Assuming that secretion takes place across the entire pulmonary epithelium: $J_v/A_L = 15/2{\cdot}8 \times 10^4 = 0{\cdot}54$ μl. hr^{-1} cm^{-2}. If, on the other hand, the property of secretion is restricted to the tracheobronchial epithelium (see below), and if the tracheobronchial epithelium constitutes between 1 % and 5 % of the total surface area of the lung, the corresponding value for net water flux would be between 54 and 10·7 μl. hr^{-1} cm^{-2}.

Whereas the change in lung liquid volume with gestation appears to be more or less linear over the period studied, γ, secretion rate per unit of lung liquid volume, is initially high at 70 days and then falls precipitously to reach a plateau at about 95 days (Fig. 1). This period of gestation corresponds to a stage of lung development in which important changes in structure are taking place. The tubular channels distal to the carena, which at 70 days are scanty and lined by columnar epithelium, show a marked increase in number, whilst the epithelium becomes cuboidal (Schneeberger *et al.* 1978). Morphological changes such as these can be linked with the decline in γ only if we postulate that the increase in V_L is not matched by a concomitant increase in secretory capacity (since $\gamma = J_v/V_L$). This could arise if the property of lung liquid secretion resides only in the proximal airways or if the change from a columnar to a cuboidal epithelium is associated with a decrease in secretory activity per unit area.

Permeability measurements and epithelial tight junction strand number

Using pore theory to describe the permeability of the lung epithelium to small water-soluble non-electrolytes, we have found that low solute permeability is already a feature of the pulmonary epithelium early in gestation and in this respect the immature lung of 69–76 days is no different from the lung of the mature fetus near term. However, such an analysis of the data, though adequately representing the

quantitatively important aspects of permeability, fails to take into account the fact that inulin (1·39 nm molecular radius) penetrates the immature lung epithelium (equivalent pore radius , 0·66 nm) to a small but significant extent, the degree of penetration declining with increasing gestational age (see Methods). This apparent anomaly could be accounted for by the presence, in addition to the small-pore system, of large pores or leaks in the lung epithelium, the proportion of which diminishes with advancing gestational age. The existence of large pores ($r \approx 3{\cdot}5$ nm) has been reported in the airway epithelium of the adult rat lung but none have been found in alveoli (Gatzy & Stutts, 1979). If the same were true of the fetal lung we would expect inulin penetration to be greatest early in gestation when the major part of the lung epithelium is that which lines the potential airways. Although we are unable to define the size of the 'leaks', we can be sure that they are limited in number since plasma proteins, including albumin, are severely restricted by the epithelium, as shown by the fact that lung liquid protein concentration remains very low ($< 0{\cdot}35$ g $l.^{-1}$) throughout gestation in the face of a plasma protein concentration which rises from 23 g $l.^{-1}$ at 69–76 days to 43 g $l.^{-1}$ near term.

The limited degree of inulin penetration apart, the pulmonary epithelium is functionally 'tight' to non-electrolytes at all gestations while, if it changes at all, permeability to electrolytes falls during this period (see below). Yet between 69 and 140 days' gestation we have observed a halving of the mean strand number of the epithelial tight junctions. If there were a simple inverse relationship between strand number and permeability of the kind suggested by Claude & Goodenough (1973), we might have expected to see a concomitant increase in equivalent pore size and an increase in permeability to ions. Clearly no such simple relationship exists in the epithelium of the developing lamb lung (nor does it exist in the capillaries of the developing brain: Dziegielewska, *et al.* 1979). This lack of correlation between strand number and permeability is perhaps not so surprising in the case of ions where fixed surface charges may play an important role in determining molecular transfer across the epithelium (see below). Furthermore, it is conceivable that in fetal tissues tight junction strands may have functions other than those concerned with permeability; they may have special importance, for example, in assuring intercellular adhesion during a period of rapid growth and development.

Ion fluxes and transport e.m.f.

A striking finding in the immature fetal lung of 84–87 days' gestation, the earliest time at which we could make such measurements, is that the transfer constants (K_0, min^{-1}), for sodium and chloride are, respectively, some 6 and 4 times greater than in the fetus near term (at a time when the diffusion path length, dx, is much reduced: see eqn. (4) in Methods). A simple reduction in the area-to-volume ratio of this magnitude seems unlikely and in any case could not explain the fact that the values of K_0 for sodium and chloride decrease by different amounts. Furthermore, the non-electrolyte K_0 values fall by less than either. These observations imply that an alteration in ion–membrane interaction takes place during development, perhaps as a result of changes in epithelial fixed charges. Such a possibility could be confirmed by the measurement of epithelial permeability to other alkali metals and halides (Diamond & Wright, 1969; Olver & Strang, 1974).

As in the mature fetal lung, the pulmonary epithelium at 84–87 days appears actively to transport chloride into the lung lumen (transport e.m.f., +20·5 mV). While there is no positive evidence of active sodium transport (transport e.m.f., −1·1 mV) we cannot exclude this possibility. The reason for our uncertainty arises from the fact that the transport e.m.f. is only a measure of the net force acting on an ion causing it to be actively transported so long as exchange diffusion remains a quantitatively unimportant factor (see Methods, and Ussing & Zerhan, 1951). The greater the exchange diffusion in relation to other transport processes, the closer will the ratio of the one-way fluxes (J_{PL}/J_{LP}) approach 1·0. Hence, transport e.m.f., which is a means of expressing the difference (as a voltage) between the observed flux ratio and that predicted on the basis of the measured forces, will tend to be underestimated.

Transport e.m.f. is calculated from measurements of potential difference, K_0, γ and ion activity ratio (Table 5). Since the plasma/lung liquid activity ratio for chloride remains constant throughout gestation, its transport e.m.f. at different gestational ages will depend upon the magnitude of K_0 relative to γ, and the measured potential difference (see eqn. (3) *et seq.* in Methods).

In spite of the fact that the K_o values for chloride (Table 5) early in gestation are substantially higher than they are near term, the effect on transport e.m.f. is largely offset by the high values of γ (Fig. 1). Thus, although the transport e.m.f. for chloride is significantly lower in the immature group than the value obtained by Olver & Strang (1974) close to term, the difference (5·5 mV) is not large and would lose statistical significance if our measured values of potential difference were too low by as little as 2 mV. Although the figure used for calculation of transport e.m.f. was that recorded with the tip of the lung lumen potassium chloride–agar bridge situated 1–2 cm proximal to wedging, as was the case in the study of Olver & Strang (1974), potential differences 2–3 mV greater (i.e. more negative) were regularly seen with the tip of the potassium chloride–agar bridge some 4–5 cm proximal to this point (a phenomenon not observed in mature fetuses). Anatomically these positions usually corresponded to a third- to fifth-generation bronchus and main bronchus respectively.

Plasma and lung liquid electrolyte composition

Whereas sodium appears to be passively distributed across the lung epithelium at all gestations, with a plasma/lung liquid concentration ratio near unity, the potassium concentration in lung liquid is consistently higher than in plasma, a finding which has been shown, in the mature fetus, to be due to active potassium secretion (Olver & Strang, 1974). In the few days immediately before delivery at term, there is an abrupt rise in lung liquid potassium concentration, thought to be associated, at least in part, with surfactant release (Mescher *et al.* 1975).

The most straightforward explanation for the increase in lung liquid chloride concentration with gestation is that it merely follows the plasma concentration, the chloride transport mechanism maintaining a plasma/lung liquid concentration of approximately 0·7 at all times while both the K_0 for chloride and γ fall with advancing maturity. Total anion concentration within the lung liquid remains more or less constant because, as the concentration of chloride in lung liquid rises, that of bicarbonate falls. Plasma bicarbonate concentration remains constant throughout gestation and thus the bicarbonate plasma/lung liquid ratio rises from 3 at 70 days

to 20 near term. Olver & Strang (1974) have put forward evidence to show that in late gestation the high plasma/lung liquid ratio for bicarbonate is the result of active transport, either of bicarbonate from lung lumen to plasma or hydrogen ions in the reverse direction. It follows that the rise in plasma/lung liquid ratio for bicarbonate during gestation could be due to maturation of this active transport system. This maturation could involve both more efficient coupling of the actively transported ion with its carrier system and diminished passive back-flux of the ion as epithelial permeability to electrolytes falls.

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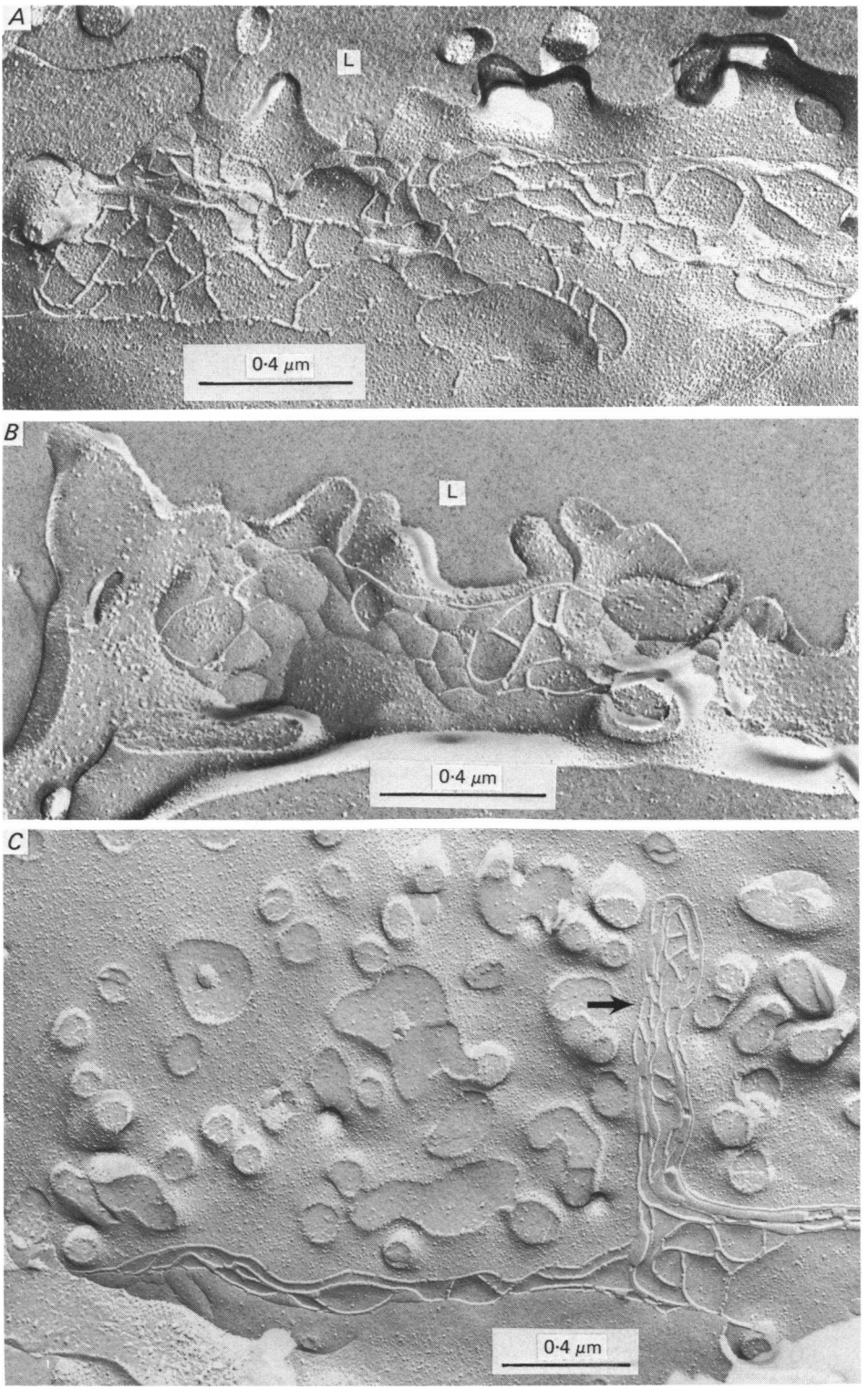
EXPLANATION OF PLATE

Freeze-fracture replicas of epithelial tight junctions from lungs of fetal lambs. L denotes the lumen of the lung in micrographs *A* and *B*.

A, At 70 days' gestation the tight junction consists of a network of five to eight interconnecting strands. × 62,000.

B, at 142 days' gestation the tight junction between two type I pneumocytes consists of a network of three to five interconnecting strands on the protoplasmic face with complementary grooves on the exoplasmic face. × 67,000.

C, a tight junction between type I and type II pneumocytes from the same animal as in *B*. The junction comprises a network of two to four parallel strands on the protoplasmic face with complementary grooves on the exoplasmic face. A loop of junctional strands (arrowed) projects towards the luminal border of the type II pneumocyte. The alveolar lumen is above the top edge of the micrograph. × 51,000.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