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FLUID ABSORPTION BY RAT LUNG *IN SITU*: PATHWAYS FOR SODIUM ENTRY IN THE LUMINAL MEMBRANE OF ALVEOLAR EPITHELIUM

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SUMMARY

1. The purpose of the investigation was to characterize the luminal membrane and the paracellular pathway of rat lung alveolar epithelium. Experiments were performed on lungs *in situ* instilled with isotonic, buffered Ringer solution and perfused with blood from a donor rat using cross-circulation technique.

2. The rate of active Na^+ transport was 4·4 pmol/(cm² s). The fluid absorption was 156 nl/s, and was unaffected by the presence of protein in the instillate (166 nl/s). In the absence of Na^+, fluid absorption was zero. Amiloride (10^{-3} M) reduced fluid absorption by 60%. Amiloride, combined with absence of D-glucose, arrested fluid absorption completely. Phloridzin at the luminal side reduced fluid absorption whilst phloretin had no effect. Amiloride together with phloridzin (10^{-3} M) also arrested absorption. Thus, there are two entry systems for Na^+ in the luminal membrane: Na^+ channels and a Na^+–D-glucose symport. These results show that alveolar fluid absorption is due to cellular activity.

3. Substitution of Cl^- with gluconate not only stopped fluid absorption, but led to slight reversal of net fluid movement.

4. Passive unidirectional flux of Na^+, determined with $^{22}Na^+$, was 9·9 pmol/(cm² s) and that of Cl^-, determined with $^{36}Cl^-$, was 12·4 pmol/(cm² s). These fluxes were based on an assumed alveolar surface area of 5000 cm². Transference numbers calculated from these figures are close to those in free solution, suggesting a neutral or weakly charged intercellular junctional pathway. The D-mannitol permeability in the paracellular pathway was $1{\cdot}7 \times 10^{-8}$ cm/s.

5. It is a consequence of the proposed mechanism for fluid absorption that it becomes inoperative if the normally high reflexion coefficients for Na^+ and Cl^- are lowered in pathological states. In such conditions pulmonary oedema may develop depending on the net balance of passive mechanical and colloid–osmotic forces.

6. An explanation of the reversal of fluid transport at the time of birth is presented.

INTRODUCTION

In the accompanying paper (Basset, Crone & Saumon, 1987) we outlined several of the characteristics of the alveolar epithelium and showed that it is a fluid-transporting structure which probably plays a decisive role in the fluid balance across

the alveolar membrane with mechanical and osmotic forces being secondary. We provided experimental evidence for the existence of a Na^+–K^+ pump located in the abluminal membrane and for amiloride-blockable Na^+ entry sites in the luminal membrane. In this paper we extend these findings, corroborate the view that fluid is moved from the alveoli to the capillaries secondary to active transport of ions, and provide evidence for two separate Na^+ entry systems in the luminal membrane. Besides the specific Na^+ channels, a D-glucose-coupled Na^+ co-transport system was identified. This observation was unexpected since D-glucose-coupled Na^+ entry is a feature that characterizes 'leaky' epithelia. Although the over-all electrical resistance of the alveolar epithelium presumably is high, it may not belong to the group of tight epithelia. We have already touched upon this problem (Basset *et al.* 1987) and shall discuss it in some greater detail at the end of this paper.

It may be of interest to know whether an isolated blood-perfused lung preparation serves as a satisfactory system to describe the alveolar epithelium under *in vivo* conditions. To answer this we carried out the present series of experiments on a cross-circulation system where the lungs *in situ*, instilled with fluid, were perfused from a donor rat. Thus the lungs were perfused with blood containing hormones and other plasma components that cannot be mimicked in an artificial perfusion system as used previously (Basset *et al.* 1987).

The main difference between the two systems was a higher rate of fluid transport rate in the cross-circulated lung preparation reflecting a better physiological condition and more homogeneous perfusion, but on the whole the results comply with those obtained on pump-perfused lungs.

The experimental strategy employed in the present experiments follows that in the accompanying paper, except that ouabain experiments cannot be performed on lungs perfused with the heart as a pump. Apart from describing the D-glucose–Na^+ co-transport system and the observation that omission of Na^+ from the instillate completely blocks fluid absorption, we characterize the paracellular pathway and throw additional light on the nature of the Cl^- transport that accompanies the Na^+ transport.

Having identified an active fluid transport two questions arise: (1) what is the origin of the fluid that enters the alveoli and which is balanced by absorption? (2) what is the nature of the control system that ensures a proper thickness of the fluid film on the inside of the alveoli? We shall discuss these two problems at the end of the paper. We also propose a mechanism that may explain the reversal of the direction of transalveolar fluid transport occurring at the time of birth.

METHODS

The present experiments were based on methods that allow fluid transport in the alveolar epithelium to be measured together with net and unidirectional ion fluxes. To approach this problem the fluid-filled alveoli were considered to constitute a well-stirred space from which water disappearance could be followed by means of an impermeable tracer (^{125}I-labelled albumin). The movements of solutes in the isotonic instillate were measured with appropriate isotopes ($^{22}Na^+$, $^{36}Cl^-$ and [^{3}H]mannitol). These isotopes were essentially lost to an 'infinite' reservoir consisting of the blood and tissues of the recipient and donor rat. Known amounts of the instillate were withdrawn and analysed at various time points after the instillation, thus allowing the development of the absorption process to be quantitatively assessed.

Many of the details of the experimental technique are described in the accompanying paper (Basset *et al.* 1987) which should be consulted for further information.

Cross-circulation technique

The lungs of the recipient rat were perfused with blood from a donor rat. Two rats were placed side by side after having been anaesthetized with Nembutal given intraperitoneally, 60 mg/kg body weight. A catheter introduced into the abdominal aorta of the donor rat led to a reservoir placed about 25 cm above the animal. From the bottom of the reservoir blood flowed into a catheter placed in the inferior caval vein of the recipient. Thus arterialized blood flowed into the right ventricle, lungs and left ventricle of the recipient. When arriving at the aorta of the recipient it was diverted into another reservoir via a catheter in the abdominal aorta. From the bottom of this reservoir it returned to the inferior vena cava of the donor, thus completing the circle. The lungs of the donor rat were ventilated from a Braun respirator (Braun, Melsungen, F.R.G.).

This experimental arrangement allowed fully oxygenated blood to reach the coronary circulation of the test rat. The cannulation as carried out left the upper part of the body normally perfused. The external circuits were thermostated at 37 °C by means of water-jackets. An important factor in a cross-circulation system is preservation of a good coronary perfusion through both hearts. This required establishment of a variable resistance in the output tubes from the two aortae by means of Starling resistors. Once this precaution is taken the experiment may continue for many hours. Slight differences between the outputs of the two hearts were adjusted by variations of the height of the blood in the reservoirs above the caval veins, determining the diastolic filling pressure and thereby the ventricular performance of each heart. The cardiac output varied between 10 and 50 ml/min. Since with this technique the liver is excluded, the plasma D-glucose concentration falls steadily. A reasonably constant plasma D-glucose concentration of about 10 mM was obtained by depositing 0·5 ml of a 50 % D-glucose solution subcutaneously in the upper part of the thorax of the donor rat. To fill the external circuit (including reservoirs) about 50 ml of blood was needed. This was obtained from heparinized donor rats.

After the cross circulation had been established the two rats were left to stabilize. Often there was a temporary discrepancy between the two cardiac outputs that subsided during the subsequent 20 min.

Composition of instillates

Bicarbonate-buffered medium (mequiv./l): Na^+, 145; K^+, 5; Ca^{2+}, 2; Mg^{2+}, 2; Cl^-, 129; HCO_3^-, 25. Phosphate-buffered medium (mequiv./l): Na^+, 142; K^+, 5; Ca^+, 2·5; Mg^+, 1·8; Cl^-, 146; HCO_3^-, 4·2; HPO_4^{2-}, 0·3; $H_2PO_4^-$, 0·4; SO_4^{2-}, 0·8. Both buffers contained 10 mM-D-glucose.

The reason for working with two buffers was to see whether the presence of bicarbonate influenced the absorption rate. The two buffer solutions were used as required by the logic of the experiments. In experiments with Na^+-free solutions Na^+ was substituted with choline, and in Cl^--free experiments gluconate was used instead of Cl^-.

Isotopes

The water movement into or out of the alveoli was determined by the concentration variations of an impermeable tracer, [^{125}I]albumin added to the instillate. A small amount of carrier albumin (rat plasma) was added in order to avoid problems with radioactive albumin sticking to the tubes or syringes. Usually 3 μCi [^{125}I]albumin (CNTS, Paris, France) was added to the instillate. To determine the permeability of the paracellular pathway 4 μCi ^{3}H-labelled D-mannitol (New England Nuclear, Frankfurt, F.R.G.) was added. Unidirectional movements of Na^+ and Cl^- were followed with $^{22}Na^+$ and $^{36}Cl^-$ (CEA, Saclay, France). Routinely 1 μCi $^{22}Na^+$ or 5 μCi $^{36}Cl^-$ was added to the instillate.

Counting was performed in a scintillation counter and in a γ-counter (Intertechnique, France). Appropriate corrections for spill-over between channels were made with standards containing only one isotope. The counting was continued until at least 10000 counts were obtained ensuring counting statistics with a S.D. below 1 %.

Technique of alveolar instillation

When the preparation had stabilized, 2 ml of air were withdrawn from the recipient lungs and 5 ml of buffered isotonic Ringer solution with appropriate isotopes were slowly introduced via the

tracheal cannula, followed by a dead-space volume of 0·5 ml air. The pressure in the fluid-filled lungs was measured in some instances and found to be less than 0·8 kPa.

After instillation a period of 10 min was allowed for osmotic equilibration. Then the first sample of fluid was taken. First, air in the dead space was withdrawn. Then a sample of about 0·5 ml was taken for analysis and air returned to the upper airways. In most experiments three successive samplings were carried out with 30 min intervals. Knowing the initial volume and the volume withdrawn for analysis (determined by weighing) together with the variations in the concentration of [^{125}I]albumin, the volume changes of the instillate could be calculated. The experiments aimed at determining the following parameters: (1) transalveolar fluid movement, (2) net flux of Na^+, (3) paracellular fluxes of labelled ions, (4) passive permeabilities of D-mannitol, Na^+ and Cl^-, and (5) rate of disappearance of D-glucose.

Knowledge of these figures allows several conclusions to be drawn regarding the fundamental characteristics of the alveolar epithelium, i.e. transcellular and paracellular ion movements. Once these elements had been characterized a series of experiments were carried out using amiloride (Coger, Paris, France), bumetanide (3-(butylamino)-4-phenoxy-5-sulphaminoylbenzoic acid, kindly supplied by Leo Pharmaceutical Products Ltd., Copenhagen), DIDS (4,4′-diisothiocyanostilbene-2,2′-disulphonic acid; Sigma, St. Louis, MO, U.S.A.), acetazolamide (Sigma), phloretin (Sigma) and phloridzin (Sigma).

Water and solute movements are formally described by differential equations that are integrated with proper reference to the boundary conditions as explained in detail in the accompanying paper by Basset *et al.* (1987); only the main components of the analysis are given here.

Methods of calculation

The analysis of transalveolar fluid movement was based on mass conservation, assuming that the mass of labelled albumin did not change during each experimental period of 30 min. If albumin does not leave the alveoli the changes in albumin concentration ($C_{o,Alb}$) are proportional to the changes of the alveolar fluid volume V_o, allowing the calculation of the rate of fluid absorption:

$$\frac{dV_o}{dt} = -\frac{V_o}{C_{o,Alb}} \frac{dC_{o,Alb}}{dt}. \quad (1)$$

To calculate unidirectional ion fluxes and D-mannitol permeability the following assumptions were made. (1) The absorbed fluid has a ionic composition similar to that of the instillate. (2) The active transport of Na^+ is defined as the amount of Na^+ contained in the absorbed volume. (3) The passive fluxes are calculated as the remaining fluxes. This means that the Cl^- flux coupled to the active Na^+ flux in principle is treated as being transcellular. (4) There is no significant osmotic pressure difference or hydrostatic pressure difference between instillate and interstitial fluid. (5) The transalveolar electrical potential difference is negligible, i.e. below 5 mV. This assumption means that the potential term of the integrated flux equation (Ussing, 1949) is left out. (6) The rate of absorption of pure water, J_w, is constant within each experimental period. Then the active ion flux due to cellular activity (J_{act}) and the flux through the paracellular pathway (J_{pass})

$$J_{act} = J_w C_o, \quad (2)$$

$$J_{pass} = PA(C_o - C_i), \quad (3)$$

where P is the permeability of the solute and A is the total surface area of the alveolar membrane, C_o and C_i are the alveolar and plasma concentration of the solute, respectively. The volume of instillate (V_o) changes with time

$$V_{o(t)} = V_A + J_w t. \quad (4)$$

Since total unidirectional Na^+ flux (J_{tot}) is

$$J_{tot} = J_{act} + J_{pass}, \quad (5)$$

it follows that, when integrated over a period of time x,

$$PA = -J_w \ln \frac{(*C_i - *C_o)_{t=x}}{(*C_i - *C_o)_{t=0}} \Big/ \ln \frac{V_A + J_w x}{V_A}, \quad (6)$$

for an actively transported tracer, and

$$PA = -J_w \left(1 + \ln \frac{{}^*C_{o,t-x}}{{}^*C_{o,t-0}}\right) \Big/ \ln \frac{V_A + J_w x}{V_A}, \quad (7)$$

for a passively transported tracer when the plasma concentration is negligible. Asterisks indicate tracer concentrations.

The difference between eqns. (6) and (7) expresses the relative increase in concentration of a passively moving solute, due to reduction of the volume of the instillate as a consequence of solute-coupled water flow.

Statistical methods

All results within groups and between groups were compared by analysis of variance. Correlations were obtained by use of the unweighted least-square method.

RESULTS

The data were analysed according to the conception of an absorbing epithelium consisting of a layer of cells in parallel with a paracellular, passive pathway. Water absorption was considered to be secondary to active transcellular Na^+ transport. The rheogenic Na^+ transport drags Cl^- and bicarbonate ions so as to create an isotonic absorbate. The nature of the Na^+ transport concords in general terms with the concept of an electrodiffusive Na^+ entry at the apical cell membrane and an active extrusion at the antiluminal membrane (Koefoed-Johnsen & Ussing, 1958).

Water absorption

The fractional reduction of volume was about 5–7 %/30 min, somewhat higher in the last period than in the first as expected for a process proceeding with almost constant rate from a shrinking volume. The average rate was 156 nl/s (S.E., 8·9; $n = 27$, where n is the number of individual periods of 30 min). In the first period the absorption rate was 175 nl/s (S.E., 12·4; $n = 11$), in the second it was 146 nl/s (S.E., 14·5; $n = 10$) and it was 139 nl/s (S.E., 20·9; $n = 6$) in the third. Statistical analysis showed these rates not to be significantly different. The problem of the effect of colloids in the instillate discussed in the accompanying paper (Basset *et al.* 1987) was further investigated in experiments where plasma was instilled. The average rate of fluid absorption was 166 nl/s (S.E., 17·9; $n = 12$), showing that with the same colloid–osmotic pressure on the two sides of the alveolar membrane absorption was not diminished (corroborating Matthay, Landolt & Staub, 1982).

Net Na^+ transport

Epithelial transport is normalized by expressing the rates per square centimetre of surface area. We have chosen Weibel's figure for the surface area of the rat lung alveoli which is close to 5000 cm^2 (Weibel, 1973) for a 300 g rat. The normalization allows comparisons with transport processes in other epithelia. The net movement of Na^+ across the epithelium was calculated as the amount contained in the isotonic absorbate. In the first period it was 25 nmol/s, in the second it was 21 nmol/s, and it was 20 nmol/s in the third period. The global average was 22 nmol/s, or 4·4 pmol/(cm^2 s) (S.E., 0·24, $n = 27$). In a short-circuited preparation this would give a short-circuit current of 0·4 $\mu A/cm^2$, if Na^+ were the only actively transported ion. This is a very low rate of active transport, compared to other epithelia.

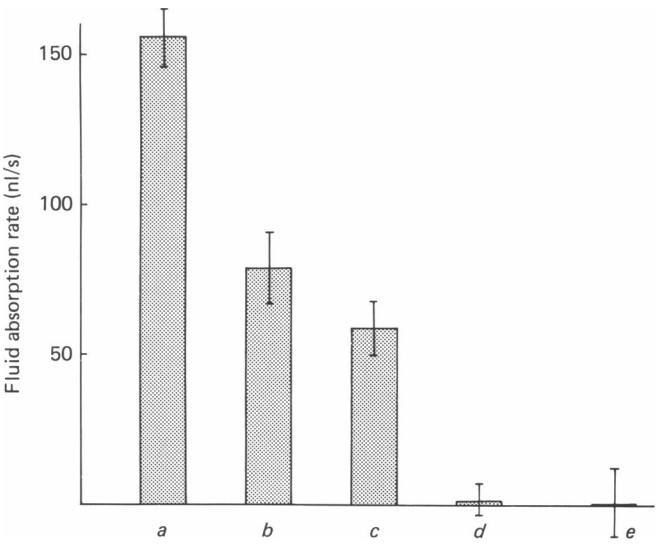

Fig. 1. Rate of transalveolar fluid absorption under various experimental conditions. *a*, control experiments; *b*, no D-glucose present in instillate; *c*, amiloride (10^{-4}–10^{-3} M) in instillate; *d*, zero D-glucose plus amiloride; *e*, Na^+ substituted with choline.

Characterization of the Na^+ entry across the luminal membrane

Amiloride effect. From analogy with findings in many epithelia an electrodiffusive entry of Na^+ is to be expected. This entry mechanism is characteristically sensitive to amiloride (Bentley, 1968). Amiloride placed in the instillate in concentrations of 10^{-4}–10^{-3} M exerted a clear effect on fluid absorption, which was reduced to 60 nl/s (S.E., 7·2; $n = 26$). Thus the reduction was about 50–60 %. Although the amiloride effect indicates the presence of Na^+ channels (Cuthbert, 1976), high doses (such as those employed) also interfere with Na^+–H^+ exchange systems (Kinsella & Aronson, 1981). It was constantly observed that the concentration of D-glucose in the instillate declined regularly, but the disappearance of D-glucose was slowed in experiments without Na^+ in the instillate. This observation initiated the following experiments.

Effect of removal of D-glucose from the instillate. Instead of D-glucose, equimolar amounts of mannitol were added to the instillate (to balance the D-glucose present in plasma). In the absence of D-glucose a clear reduction of the rate of fluid absorption was seen. The average absorption rate under these circumstances was 79 nl/s (S.E., 8·9; $n = 9$). Thus the rate of absorption went down by about 40–50 %, indicating the presence of a Na^+–D-glucose co-transport system. Since amiloride also reduced the absorption rate, two entry systems for Na^+ were likely to be present. Experiments with a combination of amiloride and absence of D-glucose in the instillate would be expected to further reduce fluid absorption due to additive effects.

Effect of amiloride and absence of D-glucose on alveolar fluid absorption. The expectation that with this combination fluid absorption would approach zero was fully borne out. The global rate of fluid absorption in these experiments was 0·1 nl/s (S.E., 6·3; $n = 15$). Thus, two Na^+ entry systems are present in the luminal membrane.

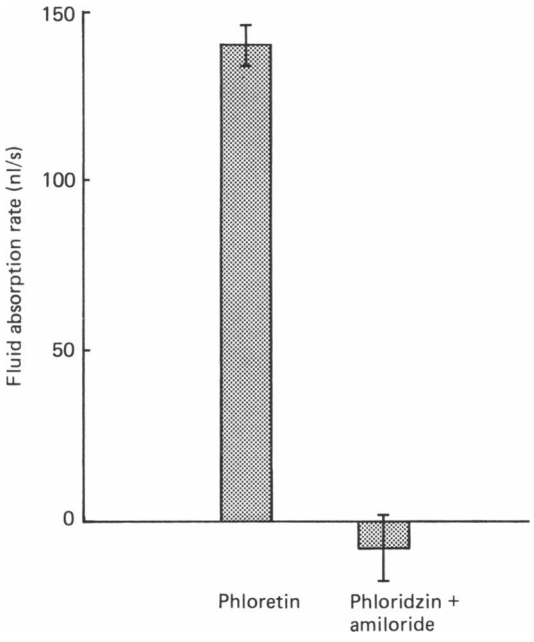

Fig. 2. Fluid absorption rate with phloretin (10^{-3} M) in the instillate is unaltered, whilst the combination of phloridzin (10^{-3} M) and amiloride (10^{-4} M) led to arrest of fluid absorption.

The effects of amiloride combined with absence of D-glucose are shown in Fig. 1 together with the earlier results.

Effect of phloridzin and phloretin on alveolar fluid absorption. Phloridzin is well known to inhibit Na^+–D-glucose co-transport (Toggenburger, Kessler & Semenza, 1982) and the existence of a Na^+–D-glucose symport was corroborated in experiments where phloridzin (10^{-3} M) was present together with amiloride (5×10^{-4} M). Under such conditions fluid movement was effectively stopped and even slightly reversed, being 7·8 nl/s (S.E., 10·1; $n = 9$) in the opposite direction (Fig. 2). The view that D-glucose entry is coupled with Na^+ entry was further substantiated in experiments with phloretin which is known to affect facilitated but not Na^+-coupled transport (Kinne, Murer, Kinne-Saffran, Thees & Sachs, 1975). Phloretin (10^{-3} M) in the instillate did not affect fluid absorption, the average fluid absorption rate being 140 nl/s (S.E., 6·4; $n = 9$).

Dependence of the fluid transport process on Na^+. According to the experiments described so far, Na^+ is necessary for fluid absorption. The most efficient way of demonstrating this would be by removing Na^+ entirely from the instillate. Substitution of Na^+ with choline had a dramatic effect, virtually reducing the water absorption to zero. The average rate of absorption was 0·6 nl/s (S.E., 11·7; $n = 9$). The experiments unequivocally demonstrated that with no Na^+ there is no absorption of fluid, cf. Fig. 1.

'Active' ion fluxes. Table 1 shows the calculated active net fluxes of Na^+ in various experimental designs. The division of the influx of Na^+ between the two entry systems

TABLE 1. Active Na^+ fluxes (J_{Na})

	J_{Na} (pmol/(cm² s))	S.E.	n
Control	4·4	0·24	27
Amiloride	1·7	0·20	26
Zero glucose	2·3	0·39	9
Zero glucose + amiloride	0·004	0·18	15

In this and in the following tables the calculations were based on an assumed alveolar surface area of 5000 cm². n is number of 30 min periods.

in the luminal membrane is such that about 60 % is via amiloride-blockable channels and about 40 % via the Na^+–D-glucose coupled system.

Substitution of Cl^- with gluconate

The specificity of the Cl^- pathway was examined in experiments where Cl^- was substituted with gluconate. This led not only to complete arrest of fluid absorption, but to reversal, the net flow of fluid into the alveoli in these experiments being 59·0 nl/s (S.E., 10·8; $n = 12$).

It was speculated that the reversal of fluid transport might imply activation of luminal Cl^- channels following the depolarization of the luminal cell membrane in response to removal of Cl^- from the instillate (Larsen & Rasmussen, 1982). If this were the case net entry of fluid would be a consequence of Cl^- movement through the cells towards the alveoli ('secretion'). This hypothesis was tested in experiments in which a supposed abluminal co-transport system was blocked with bumetanide (10^{-4} M) in the blood perfusing the lungs. There was, however, no effect of bumetanide on fluid movement, the average rate of fluid entry into the alveoli being 61·3 nl/s (S.E., 10·9; $n = 6$).

Other attempts to affect cellular Cl^- transport systems were ineffective as well; neither DIDS (10^{-3} M) placed in the instillate or in the perfusate affected fluid movement (159 nl/s), nor did acetazolamide (10^{-3} M) in the perfusate produce any significant change (130 nl/s: S.E., 17·9; $n = 10$).

D-glucose disappearance from the instillate

The rate of D-glucose disappearance was 0·68 pmol/(cm² s) (S.E., 0·036; $n = 27$) in the control series. However, in Na^+-free instillates D-glucose disappearance fell to 0·27 pmol/(cm² s). The Na^+-free experiments agree with a Na^+-coupled system for D-glucose entry into alveolar epithelium. Fig. 3 shows that phloridzin blocks D-glucose disappearance from the instillate whilst phloretin is without effect. This implies that the only mechanism of cellular removal of D-glucose from the alveoli is by means of Na^+-coupled entry. D-glucose disappearance was, as expected, unaffected by amiloride (0·76 pmol/(cm² s): S.E., 0·036; $n = 26$).

At the end of experiments the D-glucose concentration fell to values of about 3–4 mmol/l despite the fact that the D-glucose concentration in the plasma remained between 7 and 10 mmol/l. The rate of passive D-glucose entry from plasma into alveoli under a maximal concentration difference of 10 mmol/l may be assessed.

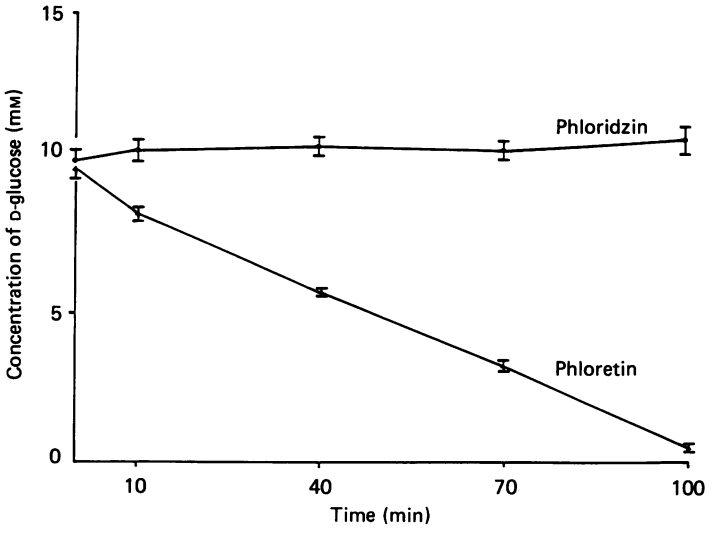

Fig. 3. The concentration of D-glucose in the instillate as a function of time. With phloretin present the D-glucose concentration falls at the same rate as in control experiments, whilst phloridzin completely blocks glucose disappearance through the luminal cell membrane.

TABLE 2. Passive permeabilities of Na^+, Cl^- and D-mannitol in the alveolar membrane

	Na^+			Cl^-			D-mannitol		
	P (cm/s $\times 10^{-8}$)	S.E.	n	P (cm/s $\times 10^{-8}$)	S.E.	n	P (cm/s $\times 10^{-8}$)	S.E.	n
Control	7·0	0·61	18	9·6	0·86	9	1·7	0·20	18
Amiloride	3·7	0·38	20	4·1	0·35	6	0·8	0·23	26
Zero glucose + amiloride	3·1	0·09	9	4·3	0·46	3	0·6	0·19	15

Assuming that D-glucose has the same permeability as mannitol ($\sim 10^{-8}$ cm/s), the flux into the alveoli (cf. eqn. 3) would be $(10^{-8}$ cm/s$) \times (10\ \mu$mol/cm$^3) = 0{\cdot}1$ pmol/(cm^2 s), which is too small to affect the calculation of D-glucose disappearance. Since the rate of entry of D-glucose into the alveoli from the blood across the paracellular pathway is so slow relative to the rate of removal by the epithelium, it is likely that in the alveolar film the concentration of D-glucose would normally be very low. This, on the other hand, would imply that about half of the Na^+ entry process would be inoperative, and the absorption rate under natural conditions lower than observed here, except if D-glucose under special conditions leaked into the alveoli at a higher rate.

D-mannitol permeability in the paracellular pathway

The existence of a paracellular pathway was demonstrated by transalveolar passage of D-mannitol. D-mannitol was included in all experiments to provide a base line characterization of this pathway. Any deviation of the D-mannitol permeability from normal would indicate changes in the paracellular route. The average D-man-

nitol permeability was $1{\cdot}7 \times 10^{-8}$ cm/s (Table 2). Wangensteen, Wittmers & Johnson (1969) determined the L-glucose permeability in rabbit lung experiments to be $2{\cdot}5 \times 10^{-8}$ cm/s, agreeing with the present figure for D-mannitol. The D-mannitol permeability was reduced somewhat in experiments with amiloride, and also in combined experiments with amiloride and absence of D-glucose (Table 2). We found a similar effect on the permeability of Na^+ and Cl^- suggesting that the presence of amiloride (with its secondary effects on cell function and, perhaps, cell volume) entails a change in 'pore' characteristics. The reduction is unlikely to be due to reduced solvent drag because, as will be shown presently, the solvent drag term is negligible compared to the pure diffusion term.

We now turn to the question of the extent of the paracellular movement of the principal ions Na^+ and Cl^-. The unidirectional fluxes of Na^+ out of the alveoli can be separated into two fluxes, an active and a passive one, the passive flux considered to be purely paracellular. The passive flux consists of two elements, an electrodiffusive flux and a solvent-coupled flux

$$J_{tot} = PC_0 + \frac{zF\Delta\psi}{RT} PC_0 + J_V(1-\sigma)\,\overline{C} + J_{act},$$

where J_v is the net water absorption, P is the passive permeability, σ is the reflexion coefficient for Na^+, C_0 is the concentration in the instillate, $\Delta\psi$ is the difference in electrical potential across the epithelium and $\overline{C}$ is the average concentration in the membrane. z, F, R and T have their usual meaning.

Thus, subtraction of the active Na^+ flux from the total Na^+ flux gives the passive unidirectional, paracellular flux. For an ion with a reflexion coefficient of 0·7 (Taylor & Gaar, 1970) and in the presence of a small electrical potential difference of 2 mV across the epithelium the various components of the passive unidirectional flux can be calculated (assuming a permeability coefficient of 7×10^{-8} cm/s). The passive diffusional flux ($P \times C_0$) is $(7 \times 10^{-8}) \times (0{\cdot}14 \times 10^{-3}) = 9{\cdot}8$ pmol/(cm² s). The electrodiffusive flux ($P \times C_0 \times zF\Delta\psi/RT$) is $9{\cdot}8 \times 0{\cdot}074 = 0{\cdot}7$ pmol/(cm² s). The flux due to solvent drag is $(150 \times 10^{-6}/5000) \times (1-0{\cdot}7) \times (0{\cdot}14 \times 10^{-3}) = 1{\cdot}3$ pmol/(cm² s). This shows that, given reasonable figures, the potential-dependent and solvent drag terms are small relative to the simple diffusional flux. The solvent drag term is probably even lower since the reflexion coefficient in reality is larger than 0·7 (see later).

The paracellular fluxes of Na^+ (J_{Na}) and Cl^- (J_{Cl}) were determined under control conditions and under various influences as summarized in Table 3.

It appears that what was observed in the experiments on isolated pump-perfused lungs (Basset *et al.* 1987) was confirmed on the present blood-perfused preparations, namely that the paracellular flux is about twice the active flux.

The transference numbers T for Na^+ and Cl^- may be calculated from the passive fluxes given in Table 3: $T_{Na} = J_{Na}/(J_{Na}+J_{Cl})$. They are 0·49 for Na^+ and 0·51 for Cl^-, compared to 0·4 and 0·6 in free solution, stressing that the charge effect in the pores is small; this was also found by Mandel & Curran (1972) in the tight frog skin epithelium.

The permeability of Na^+ and Cl^- in the paracellular pathway

The preceding calculation shows that when only a small transalveolar electrical potential is present and with a modest contribution from the solvent drag, pure

TABLE 3. Unidirectional paracellular, passive fluxes of Na^+ and Cl^- in alveolar epithelium from alveoli to plasma

	Na^+			Cl^-		
	J (pmol/(cm^2 s))	S.E.	n	J (pmol/(cm^2 s))	S.E.	n
Control	9·9	0·87	18	12·4	1·15	9
Amiloride	5·3*	0·54	20	5·4	0·46	6
Zero glucose	4·6	0·23	9	n.d.		
Zero glucose + amiloride	4·5	0·14	9	6·5	0·45	6

The fluxes were measured with $^{22}Na^+$ and $^{36}Cl^-$
n.d. = not determined.

diffusion dominates; thus, a permeability can be calculated directly from the unidirectional, passive fluxes from alveoli to blood. The values are given in Table 2. It is seen that the results agree very well with the figures obtained on isolated pump-perfused lungs (Basset *et al.* 1987). Again, the Cl^- permeability was somewhat higher than the Na^+ permeability, almost corresponding to the ratio of the diffusion coefficients. Both the Na^+ and the Cl^- permeabilities were slightly higher than calculated from the D-mannitol permeability. The ratio of the diffusion coefficients (Na^+:D-mannitol) is 2·0 in free solution; the ratio of permeabilities was 2·5 in the present experiments. This slight difference may be a consequence of the very small equivalent pore size in alveolar epithelium – 0·6 nm, according to Normand, Olver, Reynolds, Strang & Welch (1971). Such a small pore is likely to create relatively larger diffusion restriction for D-mannitol than for Na^+ and Cl^-.

A reduction of Na^+ and Cl^- permeabilities to about half the control values was found in experiments with amiloride, corresponding to the effect on D-mannitol permeability (Table 2). The explanation of this effect is not obvious, but a similar observation was made for Cl^- on short-circuited frog skin (Candia, 1978).

As mentioned, D-mannitol was included in all experiments; thus the spontaneous variation of Na^+ and Cl^- permeabilities may be compared with the spontaneous variations of D-mannitol permeabilities. The relationship is illustrated on Figs. 4 and 5, which show covariation. This implies that the variations cannot be due to spontaneous variations of transmembrane potential (that would affect D-mannitol and Na^+ and Cl^- differently). The covariation also documents that the interexperimental variations in permeability are real and not due to experimental inaccuracies. A similar linear relationship between passive ion fluxes and non-electrolyte fluxes through the frog skin was found by Mandel (1975) who showed that the variations in passive ion fluxes bore no relation to induced changes in active Na^+ transport, stressing that the phenomena took place in a 'passive' channel. The slope of a line drawn through the experimental points on Figs. 4 and 5 would be close to 2, reflecting the relationship between the ion diffusion coefficients and that of D-mannitol.

The ion permeabilities as measured were, in fact, very small, but not uniquely so. The passive Na^+ permeability is 2×10^{-8} cm/s in toad skin (Bruus, Kristensen & Larsen, 1976) and $1{\cdot}1 \times 10^{-8}$ cm/s in rabbit urinary bladder (Lewis and Diamond, 1976).

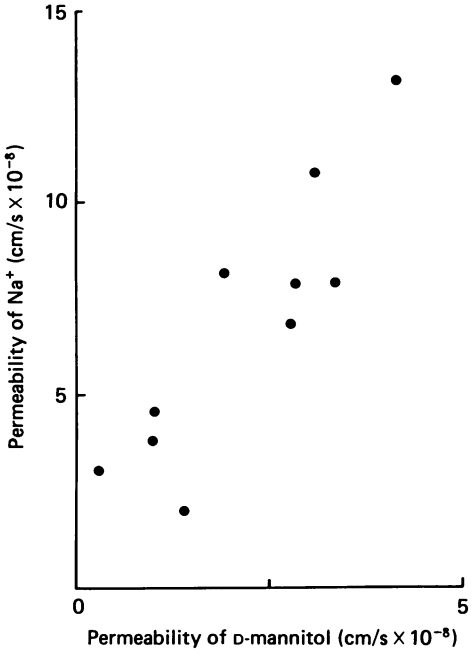

Fig. 4. Corresponding permeabilities of D-mannitol and Na^+ in ten control experiments. Based on an assumed alveolar surface area of 5000 cm^2. Each point represents data from one rat.

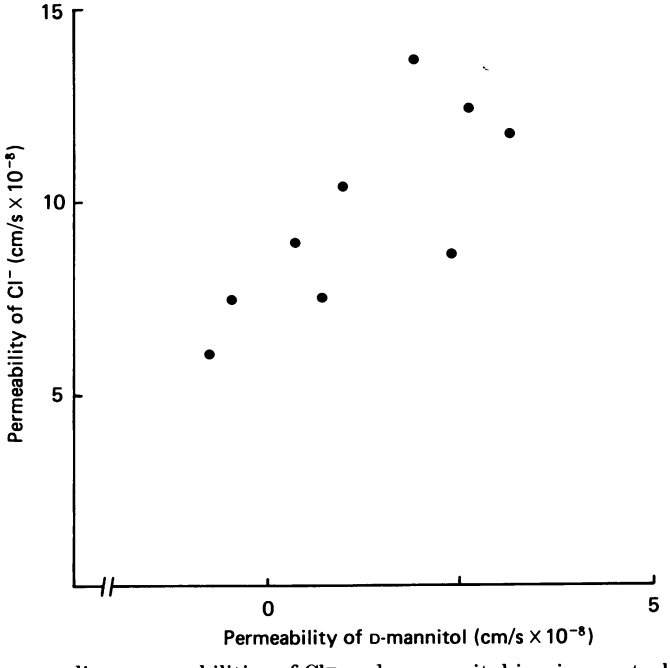

Fig. 5. Corresponding permeabilities of Cl^- and D-mannitol in nine control experiments. Based on an assumed alveolar surface area of 5000 cm^2. Each point represents data from one rat.

DISCUSSION

The intention of the present work was to provide a functional and quantitative description of the alveolar epithelium *in vivo*. Whilst the fluid transport in the alveolar epithelium of the fetus is well characterized through the work of Olver & Strang (1974) the transport processes in the epithelium of the adult lung are only vaguely known. The reason why the adult alveolar epithelium has only recently begun to be considered as a regular water-absorbing epithelium (Mason, Williams, Widdicombe, Sanders, Misfeldt & Berry, 1982; Goodman, Fleischer & Crandall, 1983) is probably that views on transalveolar fluid balance have been dominated by the supposed effects of passive, mechanical and colloid–osmotic forces. This is clearly expressed in the paper by Matthay *et al.* (1982), who observed amiloride-blockable fluid absorption from the alveoli, but hesitated to draw the full conclusions.

In the light of the present results and those of the earlier work (Basset *et al.* 1987) the conclusion is inescapable: fluid absorption from the alveoli is secondary to active Na^+ transport as in other absorptive epithelia.

We have summarized the findings of fluid and ion transport in alveolar epithelium in Fig. 6 which gives the background for the following discussion.

The luminal membrane

The key observation that substantiates that active ion transport is of prime importance for fluid transport across the alveolar wall is the arrest of transalveolar net fluid movement in response to Na^+-free solutions and to various chemical influences that are known to interfere with Na^+ transport. Thus, with a combination of amiloride and absence of D-glucose in the alveolar instillate, net fluid movement was brought to zero. This observation implies that two separate systems for Na^+ entry exist in the luminal membrane: an amiloride-sensitive Na^+ entry and a glucose-coupled electrodiffusive entry in a co-transport process known from the proximal kidney tubule and intestinal epithelium (Giebisch, Tosteson & Ussing, 1979).

We have not been able to show whether the amiloride-sensitive flux component represents net charge displacement through conductive Na^+ channels or whether it reflects a neutral Na^+–H^+ exchange process, known both from leaky and tight epithelia (Boron, 1983). This ambiguity is due to the fact that amiloride in the doses that we have employed interferes with both electrodiffusive entry and Na^+–H^+ exchange (Kinsella & Aronson, 1981). We can, therefore, only conclude that one or both entry systems are present. Further clarification requires experiments of a different nature. In view of the fact that the pH of alveolar fluid is low (Nielson, Goerke & Clements, 1981) we tend to agree with Goodman *et al.* (1983) that it is possible that there is a Na^+–H^+ exchange system rather than a conductive Na^+ entry. The finding of amiloride-blockable Na^+ entry is consistent with the observation of Olver (1983) that in the mature fetus adrenaline stimulates fluid absorption in a manner that can be inhibited by amiloride (10^{-4} M).

It was observed that if the instillate was buffered with phosphate so as to keep the bicarbonate concentration low, no significant increase in alveolar bicarbonate concentration took place. This may imply one of two possibilities: either there is a

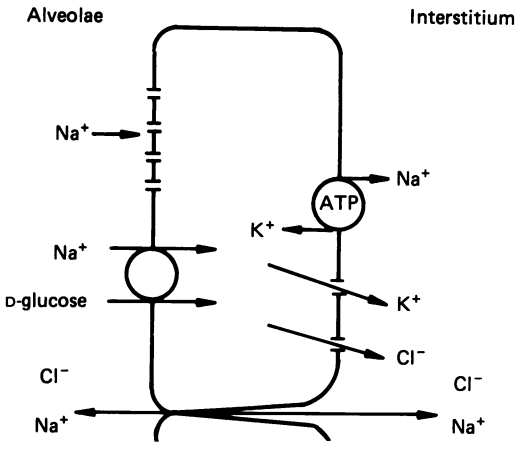

Fig. 6. Model of alveolar epithelial cell in adult rat. The luminal membrane contains two entry systems for Na^+: (1) Na^+ channels allowing electrodiffusive entry and (2) D-glucose–Na^+ symport. The abluminal membrane contains the Na^+–K^+-ATPase that pumps Na^+ into the interstitium. K^+ is recycled to keep the pump functioning. The Cl^--channel in the abluminal membrane is conjectural. The paracellular pathway is permeable to both Na^+ and Cl^-. The model does not include a luminal cellular entry step for Cl^- because we were unable to identify the nature of such a system.

high reflexion coefficient for bicarbonate (as in the proximal kidney tubules) effectively preventing bicarbonate from entering the alveoli from the interstitium, or bicarbonate which enters the alveoli due to the concentration gradient is titrated by H^+ delivered by a Na^+–H^+ exchange mechanism, and therefore the bicarbonate concentration is kept low. We were not able to distinguish between these possibilities. If what applies to the fluid secreted by the fetal alveoli holds true for adult alveoli then the bicarbonate concentration of alveolar film is low. Starting with a high bicarbonate concentration in the fetal alveolar fluid Olver & Strang (1974) observed a steady fall to values as low as a few millimolar within 4–5 h. The acid pH measured by Nielson *et al.* (1981) in adult rabbit lungs also implies a low bicarbonate concentration. The low pH may be important for the physiological control of the rate of absorption (as we shall discuss below).

The presence of a coupled D-glucose–Na^+ entry system was, of course, unexpected since such systems typically are present in 'leaky' epithelia. The observation that phloridzin (together with amiloride) leads to complete arrest of fluid absorption further supports the existence of a coupled system for Na^+ entry, because phloridzin is well known to interfere with the D-glucose–Na^+ symporter in the small intestine (Crane, 1968). It may be argued that D-glucose enters independently of Na^+ and that the effect is exerted via cellular metabolism rather than on the entry step, in which case omission of D-glucose or blockage of entry with phloridzin secondarily depresses the activity of the Na^+–K^+ pump. This is, however, unlikely, because D-glucose was available from the blood side throughout the experiment and the very high perfusion rates should easily supply the D-glucose needed for optimal cellular function. Goodman & Wangensteen (1982) observed that D-glucose moves faster from an alveolar instillate than L-glucose and Wangensteen & Bartlett (1984) noticed that

phloridzin (3×10^{-3} M) reduced the rate of passage of D-glucose into the vascular perfusate, whilst phloretin (10^{-3} M) had no effect. This fits exactly with what is reported in the present experiments and with what would be expected from a coupled Na^+–D-glucose transport system in the luminal membrane, although the authors did not draw this conclusion.

The paracellular pathway

Freeze-fracture studies of fetal and adult alveolar epithelium demonstrate the presence of typical intercellular ridges and grooves in significant numbers (Schneeberger, 1979; Olver, Schneeberger & Walters, 1981).

D-mannitol passage from instillate to plasma shows that the junctional zones are permeable. A mannitol permeability of $1{\cdot}7 \times 10^{-8}$ cm/s is in the lower range among epithelia, and reflects a well-preserved integrity of the alveolar membrane. It is often found that the junctional pathway in leaky epithelia shows some selectivity to cations, although this is not very pronounced in most cases (Giebisch *et al.* 1979). Some tight epithelia have pores that are neutral or weakly charged as seems to be the case in the alveolar membrane. This criterion is however not important enough to decide whether the alveolar epithelium should be classified as 'leaky' or 'tight'.

Whilst it is clear that Na^+ moves through the alveolar epithelium via the cells as well as paracellularly, this is less certain as regards Cl^-.

Cl^- pathways

Our experiments do not permit unambiguous conclusions concerning this point. Substitution of gluconate for Cl^- led to a complete arrest of fluid transport (and even a reversal). At first sight this indicates the presence of a transcellular component of Cl^- flux, but a similar effect would occur if gluconate, because of its larger size and negative charge, cannot (or only slowly) permeate through the junctions. If it were an uncharged solute it would have a permeability similar to that of mannitol, but for a relatively large charged ion such as gluconate the charge may interfere and significantly reduce the paracellular mobility. The reversal of fluid flow might be explained by a net movement of Cl^- into the alveoli due to the concentration gradient produced by lack of Cl^- in the instillate, combined with a slow rate of gluconate movement in the opposite direction. The electric potential gradient that arises under such circumstances would drag Na^+ into the alveoli together with Cl^- and equivalent amounts of water would follow.

Another proposal regarding gluconate effects has been put forward by Lewis, Butt, Bowler, Leader & Macknight (1985). They found that gluconate reduces the Na^+ permeability of the luminal membrane in toad bladder epithelium. Recently, Zeuthen, Christensen & Cherksey (1987) showed that in epithelial cells from choroid plexus absence of Cl^- increases Cl^- permeability by a factor of ten. The combination of closure of Na^+ channels and increasing Cl^- conductance would also lead to reversal of fluid transport.

The electrical conductance of the alveolar epithelium

The conductance (G) of the *paracellular* pathway is calculated according to

$$G = \frac{F^2}{RT}(P_{Na}C_{Na} + P_{Cl}C_{Cl}),$$

yielding a value of $56 \times 10^{-6}/\Omega$ cm^2, or a resistance of 18000 Ω cm^2 based on our values for passive Na^+ and Cl^- permeability. Mason *et al.* (1982) found only an over-all electrical resistance of 200 Ω cm^2 in cultured epithelium of type II cells, but it is a common observation that cultured epithelia never reach the same resistance values as intact epithelia. Ward & Boyd (1986) measured transalveolar resistance in *Necturus* lung mounted in a Ussing chamber and report an average resistance of about 600 Ω cm^2. They mention, however, that edge damage (the diameter of the preparation was only 0·3 cm) might well lead to underestimations. Three of their preparations showed values above 1400 Ω cm^2. Crandall & Kim (1981) report 1700 Ω cm^2 for the bullfrog alveolar epithelium. Perhaps this value is also an underestimation of the *in vivo* value since their mannitol permeability was ten times higher than the present figure.

Functional implications of the observations

The most important function of the alveolar membrane is to let the respiratory gases pass easily without hindrance. This presupposes an extremely thin diffusion barrier as reflected in the serious complications when fluid accumulates in the alveoli. It is self-evident that there ought to be a physiological mechanism ensuring a proper thinness of the alveolar fluid, the so-called 'film'. Mostly the control system is considered to be one in which passive forces govern the distribution of fluid across the membrane (Guyton, Moffatt & Adair, 1984). The present observations, together with those presented in the accompanying paper (Basset *et al.* 1987) and several other recent studies (Mason *et al.* 1982; Matthay *et al.* 1982; Goodman *et al.* 1983) stressing solute-coupled fluid transport in pulmonary epithelium, show that passive forces are unlikely to account for the stability of the film thickness. It might be speculated that the falling concentration of D-glucose in the instillate leads to a successively increasing osmotic driving force towards the interstitium. The inability of this mechanism to account for the bulk fluid movement is clearly documented by the steadily decreasing rate of fluid absorption, which was highest in the first experimental period where there was no concentration difference for D-glucose across the alveolar membrane.

The central feature is to be found in the virtual impermeability of the membrane, i.e. its very low ionic permeability. Taylor & Gaar (1970) report reflexion coefficients for Na^+ (and Cl^-) of 0·7. In view of the present findings of ion permeabilities rather close to 10^{-7} cm/s, reflexion coefficients closer to 1 are more likely. Strang (1976) calculates a reflexion coefficient of 0·9 for Na^+ in fetal alveolar epithelium and Crandall & Kim (1981) give a value of 0·99 for sucrose. With reflexion coefficients close to 1 the entire solute content of plasma and interstitium becomes fully osmotically effective. The oncotic pressure of 3·6 kPa of plasma proteins should be compared with the osmotic pressure of 1 mosmol solution which is about 2·4 kPa. This means that the maximal pressure difference that may conceivably exist across the alveolar epithelium corresponds to a concentration difference of small ions that is below the limit of detection. This is probably the explanation why the presence or absence of colloids in the instillate does not affect the rate of fluid absorption (Basset *et al.* 1987). The consequence of this is that differences in concentration of colloids in the interstitium and in the alveoli are of very minor importance for the fluid equilibrium across the alveolar wall.

The oncotic pressure difference that is so important for fluid equilibrium across capillary walls is only important by virtue of the fact that the reflexion coefficient of the proteins in most capillaries is close to 1 (Taylor & Granger, 1984) whilst that of the small ions is between zero and 0·1 (cf. review by Crone & Levitt, 1984). Only in membranes where colloid osmotic forces (or hydrostatic pressure differences) can produce *bulk movement* of fluid is the Starling principle directly applicable. With an equivalent pore radius of 0·6 nm, bulk movement of all constituents in a plasma ultrafiltrate is certainly impossible.

Having relegated Starling forces to playing a secondary role as determinants of fluid distribution across the alveolar membrane it should be added that these arguments only apply to the normal situation. As soon as the alveolar epithelium becomes damaged and the epithelial cells separate so that the reflexion coefficients of small solutes fall towards zero, the situation changes fundamentally. Now the cellular ion pump becomes ineffective relative to the mechanical forces which, because of the reduced reflexion coefficient for small solutes, now may produce bulk fluid movement. This interpretation of pulmonary oedema is consistent with the mechanism proposed by Yablonski & Lifson (1976) for fluid 'secretion' in the intestine in response to increased venous and interstitial fluid pressure. Above a certain level of interstitial pressure leakiness of intestinal epithelium occurs. Once this happens the net fluid movement is reversed because the fluid absorption of the epithelium becomes inefficient compared with the hydraulic bulk movement towards the lumen.

Is the alveolar epithelium a 'leaky' or a 'tight' epithelium?

From the foregoing discussion it emerges that this epithelium has characteristics of both types of epithelia. We mention the following features. (1) Its presumed high over-all electrical resistance would place it among tight epithelia. (2) A low rate of Na^+ transport is typical for tight epithelia. (3) The low hydraulic conductivity (Crandall & Kim, 1981) corresponds to what is expected from a tight epithelium. However, there are equally strong reasons to classify the alveolar epithelium as a leaky epithelium. (1) The higher conductance of the paracellular pathway than of the cellular. (2) The occurrence of a coupled Na^+–D-glucose transport system. (3) Solute-coupled fluid transport is a typical feature of leaky epithelia whilst tight epithelia rather separate ions and water. At present it is not possible to characterize the alveolar epithelium as belonging to one of either classes.

The fact that the passive fluxes were about two times larger than the active flux of Na^+ is not unknown for other epithelia. In the rabbit colon the passive flux of Na^+ is about 50–75 % of the total unidirectional flux (Frizzell, Koch & Schultz, 1976) and in *Necturus* gastric mucosa it is of similar magnitude (Spenney, Flemström, Shoemaker & Sachs, 1975). With a transcellular Na^+ flux of 4·4 pmol/(cm^2 s) in the alveoli and a passive flux of about 10 pmol/(cm^2 s), the relative shunt contribution is about 70 %, approaching the situation in leaky epithelia where the shunt conductance is between 85–99 % of the total conductance (Frömter & Diamond, 1972).

A 'chemical' control system

Whatever the origin of the fluid there must be a chemical control system that seems not to have been discussed before. What determines when absorption should stop? The absorption process obviously has to operate at such a rate that it leaves a film

of optimum thickness. Since it is hardly possible that the mechanical forces could counteract the Na^+ pump effectively, the control must be in the transport system itself. There are two possibilities to consider. (1) There could be a mechanism that varies the degree of opening of luminal Na^+ channels, and thus indirectly controls the rate of Na^+ pumping. The pump in the abluminal membrane seems to follow the luminal input of Na^+ (Diamond, 1982). The variable that controls the Na^+ channels could be the pH of the film. When pH reaches a certain low value it might 'titrate' the proteins in the Na^+ channels in such a way as to close them and reduce absorption. This hypothesis should be easily tested. In the event that the luminal membrane, instead of housing specific Na^+ channels (typical for tight epithelia), has a Na^+–H^+ exchange system – a possibility that our experiments do not exclude – it may again be the pH of the alveolar fluid that exerts the control. The Na^+–H^+ system is obviously sensitive to pH (Aronson, 1985) and a certain fall in pH could start a self-limiting process. This kind of operation would be effective because of the cul-de-sac nature of the alveoli. (2) Another possibility relates to the coupled Na^+–D-glucose entry system. The 'consumption' of D-glucose proceeds with such great speed that the D-glucose concentration in the alveolar film must be close to zero which means that the absorption of the Na^+ that enters the alveoli coupled with D-glucose (if interstitial fluid oozes into the lungs) could be geared to the rate of D-glucose entrance in a self-limiting way.

The two proposed 'systems' of control might thus operate on each of the two mechanisms for entry of Na^+ into the alveolar epithelial cells (cf. Fig. 6).

Explanation of the transition from a secretory fetal epithelium to an absorbing adult alveolar epithelium

Olver & Strang (1974) have shown that the fluid secretion in fetal life depends on a primary secretion of Cl^-. The absorption of fluid that must start at the moment of birth is a process that depends on active Na^+ transport (Olver, 1983). How can this transition take place? In the light of the character of the adult epithelium, we present the following hypothesis: in fetal life the Na^+–K^+ pump in the abluminal membrane extrudes Na^+ that enters via the abluminal membrane in a co-transport system together with K^+ and Cl^-. Evidence for such a coupled ion transport system driven by a Na^+ gradient has been given by Greger & Schlatter (1984) and Greger (1985). The effect is to raise the electrochemical potential of Cl^- above equilibrium. Since the luminal cell membrane in the fetal alveolar epithelium obviously contains Cl^- channels the effect would be net Cl^- flux into the alveoli with water following. If it is assumed that at the time of birth Cl^- channels are internalized whilst Na^+ channels are inserted in the luminal membrane, the direction of fluid movement will reverse, and the epithelium will change from a secretory to an absorptive one. In both conditions it is the abluminal Na^+–K^+ pump which is the 'motor'.

This proposal has an analogy in what happens when fish adapt from life in salt water to life in fresh water (Maetz, 1976). Also, the finding of Beyenbach & Frömter (1985) that a typical absorptive epithelium, such as the proximal tubular epithelium, under the influence of secretory stimuli can be converted into a secretory epithelium depends presumably on a similar set of mechanisms. Brown, Olver, Ramsden, Strang & Walters (1983) propose that adrenaline may be one of the causative agents inducing the 'reversal'; one might speculate that aldosterone were another factor.

Conclusion

In conclusion we emphasize that the findings of a complete array of well-established epithelial transport parameters in the adult alveolar epithelium means that this epithelium should now be incorporated in the physiological inventory of absorptive epithelial membranes. At present it is not dealt with in treatises of epithelial function (see, for example, Giebisch *et al.* 1979). Although accessory cellular mechanisms have been identified, the 'elements' of the transport process conform essentially with the double-membrane hypothesis proposed by Koefoed-Johnsen & Ussing (1958), with an essentially K^+-impermeable and Na^+-permeable luminal membrane and an abluminal Na^+–K^+ pump situated in a membrane with a low Na^+ and a high K^+ permeability (allowing recycling of K^+), cf. Fig. 6. Like many other epithelia the alveolar membrane represents a cellular barrier between the organism and the exterior; only its hidden place and its unassuming morphological appearance have delayed its definition as a true absorptive epithelium.

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