J. Embryol. exp. Morph. 88, 365–384 (1985)

Fluid transport across the epiblast of the early chick embryo

CLAUDIO D. STERN

Department of Human Anatomy, South Parks Road, Oxford OX1 3QX, U.K.

SIMON MANNING

Department of Anatomy & Embryology, University College London, Gower Street, London WC1E 6BT, U.K.

AND JAMES I. GILLESPIE

Department of Physiology, The Medical School, University of Newcastle-upon-Tyne, Newcastle-upon-Tyne NE1 7RU, U.K.

SUMMARY

A simple method is described which allows quantitation of the rate of fluid transport across the isolated epiblast of the early chick embryo. This method consists of allowing the tissue to form spheres, which then spontaneously undergo a large volume increase. The rate of fluid uptake into the spheres can be estimated by measuring the dimensions of the spheres. Pharmacological and electrophysiological studies were performed on the spheres to determine the mechanisms of fluid transport. It was found that fluid is driven into the interior of the spheres by the osmotic gradient generated by unidirectional sodium transport and to a lesser extent by another mechanism, as yet unknown. We discuss possible candidates for this mechanism, and consider the significance of these findings to early development.

INTRODUCTION

The formation of a blastocoele, or embryonic cavity, is a general phase in the early development of higher animals which immediately precedes the process of gastrulation. In echinoderms, amphibians and mammals, formation of the blastula entails the cavitation of the embryo so that the blastomeres enclose a fluid-filled space. In birds, the great yolk mass precludes the formation of a morula, and the blastomeres are spread out in a layer over the surface of the yolk. A fluid-filled space, perhaps corresponding to the blastocoele of mammals, does form in avian embryos, and is situated between the blastoderm and the yolk. This is termed the sub-blastodermic space (see Murray, 1933; Bellairs, 1971).

New (1956) was the first to demonstrate experimentally that the early chick embryo was capable of fluid transport. He observed that blastoderms *in vitro* remove fluid from the albumen-facing side and secrete it from their endodermal surface. The sub-blastodermic fluid is therefore derived from the albumen.

Key words: fluid transport, epiblast, chick embryo, sodium transport.

Regarding the *modus operandi* of fluid transport across early embryonic epithelia, two different theories are usually invoked. One suggestion is that there is 'active' secretion of water by the blastoderm (Tuft, 1962, 1965; Tuft & Boving, 1970). The other is that water transport is passive, driven by an osmotic force (Yamada, 1933). Two mechanisms have been put forward to account for an osmotic drag of water across the early chick embryo. Wladimiroff (1926) suggested that the formation of the sub-blastodermic fluid was related to release of acid into the yolk. Indeed, it has been shown (Davy, 1863; Wladimiroff, 1926; Sharp & Powell, 1931; Shklyar, 1937; Spratt, 1947; Romanoff & Romanoff, 1949; Romanoff, 1967) that there is a pH gradient across the plane of the vitelline membrane/blastoderm, which amounts to some 3 pH units (yolk is acidic, albumen alkaline and the sub-blastodermic fluid neutral). The other proposal, suggested by Howard (1957), argues that water is dragged across the blastoderm coupled to the active transfer of sodium from the albumen to the sub-blastodermic space. The weight of the observations of fluid transport in early embryonic development falls substantially in favour of this last explanation. For example, Cross (1973) has demonstrated active transport of Na^+ and Cl^- into the rabbit blastocoele. Using electron probe microanalysis, Borland, Biggers & Lechene (1976, 1977) have demonstrated that in the presence of external sucrose only Na^+ and Cl^- increase to a major extent inside the rabbit blastocoele, in fact enough to account for net water movement. Also, estimations of maximal water flow across the blastoderm (Adolph, 1967) correlate well with values of net water flow accompanying active salt transport across a number of other epithelia. More recently, Stern & MacKenzie (1983) have demonstrated and quantified this unidirectional sodium transport across the epiblast of the early chick embryo, and showed that it is driven by Na,K-ATPase 'pumps' located at the basal surfaces of this tissue.

Certainly, the exact mechanism by which the sub-blastodermic fluid forms is still not fully understood. With this in mind we have undertaken some experiments using the epiblast of the early chick embryo in order to clarify some of the controversies. Until now, quantitation of water transport across the chick blastoderm has been unreliable as there is no enclosed space, such as occurs in mammalian blastocysts. Two methods were previously available. One, somewhat crude, relies on comparison of wet/dry weight ratios between fluid from both sides of the blastoderm. The second method relies on measured transfer of labelled (^3H_2O) water across sheets of epiblast using Ussing-type chambers (Stern & MacKenzie, 1983). In practice, this method is not reliable for establishing the mechanism of water transport since the containers on both sides of the tissue are too large for a substantial osmotic gradient to build up across the tissue. We have therefore devised a method whereby flow rates can be experimentally investigated directly. The method consists of altering the geometry of the epiblast, such that it will seal up into a sphere, with its basal surface inwards. Changes in volume of the sphere can be quantified by measuring the diameter of the sphere with time. The spheres are capable of undergoing a more than 50-fold increase in volume in 17 h, a

rate comparable to that of the rabbit blastocoele. The resulting spheres are large enough to make them suitable to be impaled with several microelectrodes, and electrophysiological investigations could therefore be performed. The method has also enabled us to investigate the effects of different inhibitors to test the various theories on water transport.

MATERIALS AND METHODS

Culture methods

For the staging of embryos we have followed Eyal-Giladi & Kochav (1976) in Roman numerals for early (preprimitive streak) stages and Hamburger & Hamilton (1951) in Arabic numerals for later stages. Hens' eggs ('Ross Rangers') obtained from Ross Poultry (South) Ltd were incubated from stage XIV to stage 3. The embryos were explanted from the yolk and vitelline membrane in Tyrode's saline. The lower (endodermal) layer (see Stern & Ireland, 1981 for details of this layer) was removed using fine tungsten needles sharpened in molten sodium nitrite. Pieces of epiblast (about 300 μm diameter) were then excised from the centre of the *area pellucida*, excluding the region of the primitive streak. In nine specimens at stage 3 the primitive streak was included in the explanted piece.

The epiblast pieces were transferred to 35 mm diameter tissue culture dishes (Falcon) containing about 1·5 ml agar/albumen/saline (DeHaan, 1960) or 1 % agar in Tyrode's saline, and 3–4 ml of the medium to be used. The 'standard' medium used comprised medium 199 (Wellcome), 5 or 10 % foetal calf serum (Gibco), 25 units ml^{-1} penicillin and 0·25 mg ml^{-1} streptomycin (from a 5000 i.u. ml^{-1} penicillin and 50 mg ml^{-1} streptomycin stock solution – Gibco) (see Bellairs, Sanders & Portch, 1978; Bellairs, Ireland, Sanders & Stern, 1981). The medium was sterilized by filtration through a Millipore type GS (0·22 μm) filter. The lid of the culture dish was sealed on using a thin layer of egg albumen to prevent evaporation, and subsequently maintained at 37 °C.

^3H_2O flux measurements

Sealed and unsealed epiblast explants of known volume and known surface area cultured for 9 h were transferred to medium (5 % foetal calf serum in medium 199) containing 20 μCi ml^{-1} tritiated water (Specific activity of stock, 5 mCi ml^{-1}, Amersham). The spheres were subsequently cultured for 7 h, the new volume calculated from the diameter, and each sphere separately transferred to a scintillation vial after thorough washing (three changes of 10 ml each) in ice cold Tyrodes' saline. The tissue was then digested with Protosol (New England Nuclear) at 37 °C for about 1 h, and 10 ml Scintran (PPO/POPOP/toluene based scintillation solution, B.D.H.) added to each vial. The resulting cocktail was neutralized with glacial acetic acid to reduce chemoluminescence artefacts, and counted in a Beckmann LS7500 automatic scintillation counter. Quenching was controlled by the Beckmann Automatic Quench Correction technique (AQC) which uses an external ^{137}Cs standard. The counting efficiency was between 46 and 52 %. 10 μl samples of medium from each dish, taken at the beginning and at the end of the incubation period, were counted in the same way, to accurately determine the concentration of radioactive water in the medium.

Studies using inhibitors

Amiloride (obtained as a gift from Merck, Sharpe & Dohme, final concentrations up to 10^{-5} M), furosemide ('Lasix' injection, Hoechst, final concentration 10^{-5} M), strophanthidin (Sigma, final concentration up to 10^{-5} M), nifedipine (Sigma, final concentration up to 10^{-5} M), monensin (Calbiochem, final concentration 10^{-7}–5×10^{-6} M), cytochalasin-B (Sigma, final concentration 10^{-6} M) were added singly or in various combinations to the culture medium in the dishes. Controls received similar volumes of the appropriate solvents (water, except for strophanthidin, nifedipine and monensin, where ethanol was used, and cytochalasin B which was dissolved in acetone).

Strophanthidin from two separate batches was used, and medium from each batch tested for activity before and after each experiment. For this purpose, the differentiation of *Xenopus laevis* neural tissue *in vitro* (Messenger & Warner, 1979; Breckenridge & Warner, 1982) was used as a biological assay for activity. A small volume of medium was added to cultures of *Xenopus* presumptive neural cells. The reduction in the number of nerve cells present after culture with respect to controls was correlated with the effects of known concentrations of the inhibitor as previously described (Messenger & Warner, 1979; Breckenridge & Warner, 1982. The assays were kindly conducted by Ms. L. Breckenridge).

Sodium, potassium, calcium and chloride depletion studies

The composition of both medium and agar were altered. For low sodium experiments, sodium was substituted with choline chloride (Sigma), potassium was substituted with NaCl or with CsCl, calcium was replaced using $SrCl_2$ (B.D.H.), whilst chloride was substituted using 100 mM-sodium glucuronate (Sigma) (2 mM-$CaCl_2$ and 2 mM-$MgCl_2$ were included in this medium). In these cases a Pannett-Compton-based saline was used instead of medium 199, and controls were grown in this saline. The low sodium medium was buffered with KH_2PO_4 and K_2HPO_4 instead of the usual sodium salts. 0·5 % foetal calf serum was included in the culture medium in all these experiments. We estimate that the low sodium solution contained about 1 mM-sodium and the low chloride medium 5 mM-chloride.

Electrophysiology

Electrophysiological studies were performed using conventional 3 M-KCl microelectrodes (resistance 2–10 MΩ) or pH-sensitive microelectrodes (see Thomas, 1978). The pH-selective microelectrodes were made from aluminosilicate glass (Clark Electromedical) and coated with tributylcholosilan vapour (Fluka). The tips of the microelectrodes were then filled with H^+ resin (Fluka) and the shanks with buffer solution (Schulthess *et al.* 1981). The pH responsiveness of the electrodes was determined by varying the pH of the fluid bathing the microelectrode tip using calibrating solutions made with foods buffers (Sigma). Typically such electrodes had resistances of 10^{10} Ω and responded with a 50–55 mV change per pH unit. The electrodes were made and tested prior to each experiment. When using pH-selective microelectrodes the specimens were impaled with two separate electrodes (one conventional and one ion-selective) and referred to the bath ground. The potential existing across the tissue, measured with the conventional microelectrode, was subtracted by the electrometer from the potential recorded with the ion-selective electrode (comprising both transepithelial potential and that due to the specific ion being investigated). The microelectrodes were connected to a W.P.I. model FD223 electrometer (effective input resistance 10^{14} Ω). The output of the electrometer was displayed on a Tektronix 5111 oscilloscope and a Bryans BS314 4-channel pen recorder. Tip potentials were measured by the method described by Purves (1981) which eliminates liquid junction potentials. In some experiments the temperature of the preparations was maintained at 37 °C by a hair dryer and a miniature bead thermistor probe, connected to a microprocessor (305–800) controlled circuit.

In addition to the inhibitors described above and various concentrations of foetal calf serum, the effects of external pH (6–9), prostaglandin E1 (1 μM), chloride-depleted medium and various sodium concentrations were investigated. Perfusion of the bath was achieved by means of two syringe needles inserted into the bath. One of the needles was connected to a container with the desired medium, whilst continuous suction was applied to the other. At least 20 ml of the new medium were flushed through at each medium change.

Analysis of results

In most experiments one or more specimens were filmed by time-lapse cinemicrophotography on Ilford PanF 16 mm film, using a Bolex H-16 camera with time-lapse attachments (Wild) fitted to a Zeiss Standard WL microscope. Bright field, transmitted light optics were used, with a ×1 Plan objective. The microscope was fitted with a Perspex chamber which was kept at 37 °C.

The remaining dishes were cultured in an incubator at 37°C, monitored regularly and finally fixed for histochemistry.

The developed films were projected onto an Apple Computer digitizing tablet, connected to an Apple][Europlus computer. The projected perimeters of the sealed explants were outlined onto the graphics tablet, and the maximum diameter, cross-sectional area, form-factor (see below) and volume were calculated using the standard Apple graphics tablet software package. The volume was corrected for the form-factor, which is a measure of the circularity of the outline of the sphere:

$$FF = C/\pi D \quad 0 \leqslant FF \leqslant 1$$

$$Vs = \frac{4\pi R_s^3\, C}{3\pi D} = \frac{4R_s^3\, C}{3D}$$

where FF = Form-factor, C = projected circumference of sphere, D = diameter of cross section of sphere, R_s = radius of cross section of sphere, V_s = volume of sphere (corrected for Form-factor).

The initial volume of fluid contained within the spheres at time 0 was calculated making the assumption of a sphere of similar surface area to that of the original circular piece of tissue as cut out from the embryo (i.e. before any distension has occurred). This was calculated thus:

$$A_c = \pi R_c^2 \tag{1}$$

$$A_s = 4\pi R_s^2 \tag{2}$$

where R_c = radius of circular piece of tissue excised, A_c = surface area of piece excised and A_s = surface area of sphere.

At no distension, $A_c = A_s$:

$$\text{therefore: } R_c = 2R_s$$

and the volume of this sphere is given by:

$$Vs = \frac{4\pi R_s^3}{3} = \frac{4\pi(R_c/2)^3}{3} = \frac{\pi R_c^3}{6} \tag{3}$$

The rate of fluid transport into the spheres is then given by the volume at time t minus the volume at time 0 (equation 3) divided by t.

RESULTS

General behaviour of the epiblast explants

Sanders & Dickau (1981) have recently shown that explants of epiblast from young chick embryos grown on glass coverslips often form 'domes' between themselves and the substrate, with the basal aspect of the tissue lining the cavity. These domes accumulate fluid in their interior. The technique described here, where the *in vitro* substrate (agar) is made non-adhesive to cells, is a refinement of this method, which allows quantitation of the rate of fluid transport owing to the more regular geometry which the explants adopt.

A few minutes after excision, the epiblast explants curl towards their basal side (Fig. 1A) until the free edges come into contact. Within about 5 h, the edges of the explant seal together. By this time the shape of the explant is a more-or-less-regular sphere. As soon as the explant seals, the volume of the enclosed space

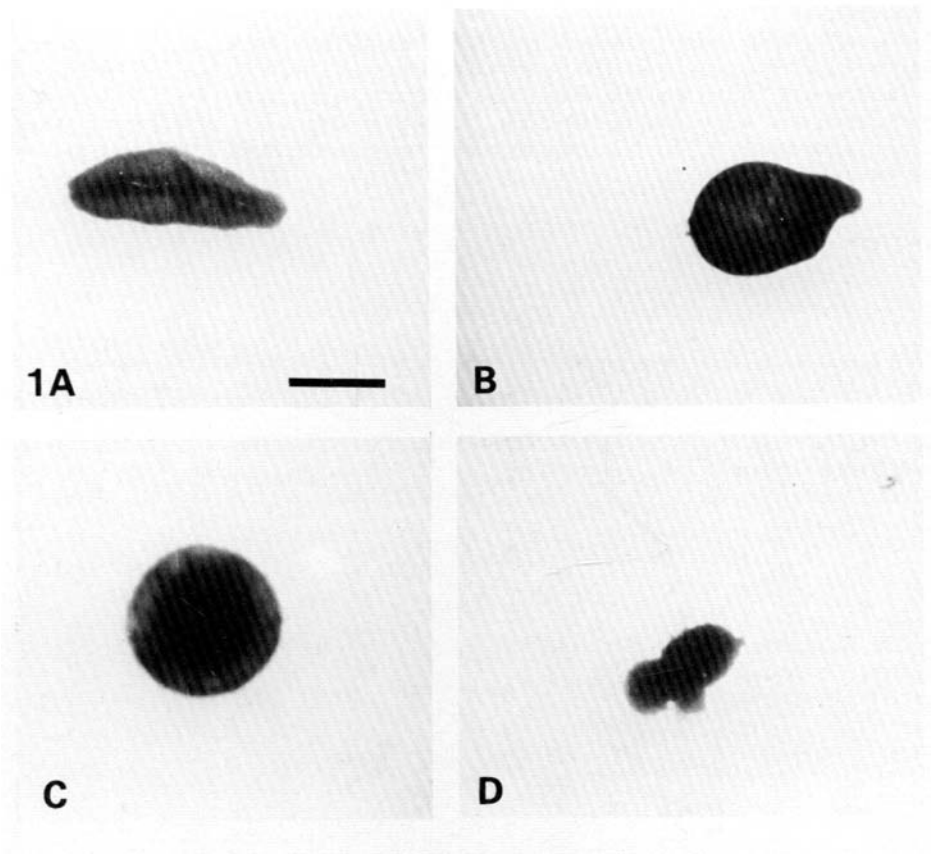

Fig. 1. Frames from a time-lapse film showing the main stages in the formation of epiblast spheres. (A) soon after explantation, the edges of the explant curl; (B) by 7 h after explantation, the edges of the explant have sealed together and the volume of the explant begins to increase, reaching a maximum (C) by about 24 h. Soon after this, (D) the sphere ruptures catastrophically, with extrusion of fluid and some cellular material. Scale bar, 500 μm.

begins to increase (Fig. 2) and the explant usually becomes a perfect sphere (Fig. 1B). Regular volume changes then take place. Each cycle lasts about 2 h (e.g. Fig. 2). After each pulsation, the volume of the sphere is slightly increased. As the sphere expands, the epiblast becomes increasingly transparent, suggesting that it stretches to accommodate the enlarging interior. The maximum volume increase we have observed was 500-fold the initial volume. A very rapid deflationary phase often follows the gradual inflation, about 20–27 h after explantation, and is generally accompanied by the extrusion of some cellular material as well as fluid (Figs 1C, 2). The minimum size reached after this phase is similar to when it had first formed. Some increase in volume and pulsations begin again soon after the deflationary phase (Figs 1D, 2), although the original maximum volume is not reached again.

Nine specimens which contained the primitive streak were also explanted. The presence of the primitive streak did not preclude sealing of the epiblast, or the increase in volume of the spheres. An interesting feature is a proboscis-like extension (Fig. 3) which forms and is gradually pinched tighter. In no case did the volume increase by more than four times the original sealed value. Only one of the explants ever underwent a rapid deflationary phase, which indicates that the internal hydrostatic pressure was rarely sufficient to cause the tissue to rupture catastrophically.

Rate of water flux

The rate of water flux in 'control' specimens was measured in the presence of 5 % foetal calf serum by two methods: calculation of volume and surface area from diameter measurements, and from ^3H_2O flux measurements. Both methods were applied to the same specimens (Table 1) and gave reasonably consistent results. We were able to establish transport rates between 1·9 and 6·8 $\mu l\,cm^{-2}h^{-1}$. Although the ^3H_2O estimates were generally lower than the corresponding estimates from measured diameter, the difference was not statistically significant (Student's paired 2-tailed t-test $P = 0{\cdot}346$ for rate comparison). Using ^3H_2O, the flux rate averaged $2{\cdot}8 \pm 1{\cdot}4$ (S.D.) $\mu l\,cm^{-2}h^{-1}$, whilst using diameter measurements it averaged $4{\cdot}4 \pm 1{\cdot}65$ (S.D.) $\mu l\,cm^{-2}h^{-1}$ in the same specimens. However, when considering volume estimates made from both sealed and unsealed explants (Table 1A and B), the difference between the two methods was found to be statistically significant at the 5 % level ($P = 0{\cdot}03$, 2-tailed test, n = 8 pairs). We suggest that this difference reflects errors in both methods: a tendency of diameter measurements to overestimate the volume (due to the thickness of the tissue, optical distortion, etc.), and a tendency of the radioactive method to underestimate (e.g. owing to loss of label during the washes). An attempt to quantify the

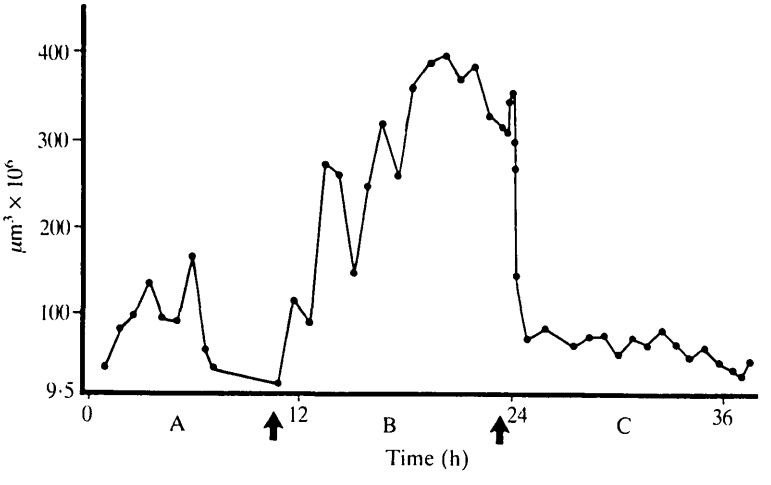

Fig. 2. Graph of volume changes during the 36 h following explantation. (A) initial oscillatory volume increase; (B) catastrophic rupture of sphere; (C) reflationary phase.

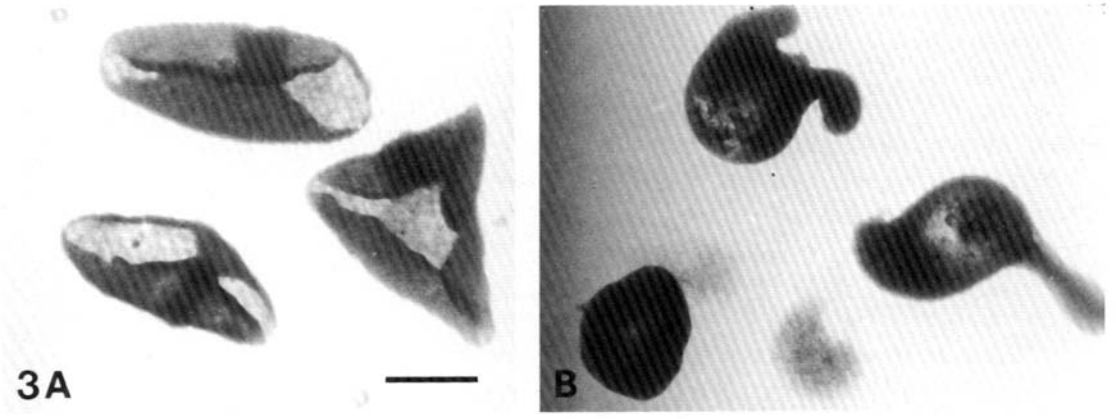

Fig. 3. Frames from a time-lapse film showing the behaviour of explants containing a primitive streak. (A) immediately after explantation; (B) 22 h later, the explants have sealed and somewhat increased in volume but have developed a 'proboscis'. Scale bar, 500 μm.

efflux of radioactivity from the spheres was unsuccessful because of the small amount of radioactivity detectable in the wash solutions.

Electrophysiological experiments

Seventeen spheres were impaled with conventional, 3 M-KCl microelectrodes to determine the transepithelial potential. Twelve of these were impaled with a single electrode, and the remaining five with two electrodes to monitor changes in sheet resistance as well as transepithelial potential. Several experiments using two of these spheres impaled with two electrodes are shown in Fig. 4. In control specimens the measured transepithelial potential was generally between the range of 2–8 mV (inside of the sphere positive), averaging +6 mV (n = 15 impalements). There were two exceptions: in one sphere, a positive potential as high as 18 mV was recorded, whilst in another a small negative potential was present, the interior of the sphere being 2 mV negative to the outside. Although the effect of temperature on the potential was not systematically investigated, we observed that the transepithelial potential could only be recorded at temperatures approaching 37°C.

The effects of several inhibitors on the transepithelial potential and resistance were investigated (summarized in Table 2). Several of the drugs were found to have significant ($P < 0{\cdot}05$) inhibitory effects on the transepithelial potential. 10 μM-amiloride reduced the transepithelial potential by 2 mV, with an increase (14 %) in transepithelial resistance. 50 μM-furosemide alone had no effect on either the potential or the resistance across the tissue, but in combination with 10 μM-amiloride it decreased the transepithelial potential by 3 mV, with no detectable change in transepithelial resistance with respect to amiloride administered alone.

5 % foetal calf serum had a stimulatory effect on the transepithelial potential (which increased by 2 mV) with respect to serum-free medium.

Sodium-free medium decreased the potential by an average of 3 mV, with no change in transepithelial resistance. Chloride-depleted medium also reduced the voltage by 3 mV, whilst inducing an increase in resistance (15 %).

Prostaglandin E1 (PGE1) increased the transepithelial potential by up to 5 mV, concomitant with a decrease in transepithelial resistance of 18 %.

A further seven specimens were impaled with a pH-sensitive electrode and a conventional 3 M-KCl microelectrode. The interior of the spheres was invariably found to be more acid (around pH 6·8) than the external solution (pH 7·5). A change of external pH from 7·5 to 9 led to a stable increase in internal pH to 8·8, which occurred within 1–2 min of the time of external pH change. Acidification of external pH from 7·5 to 6 led to a reduction in internal pH from 6·8 to 6·4. Treatment with 0·25 mg ml^{-1} furosemide alone or in combination with 10 μM-amiloride in the absence of serum led to a slow (20 min) rise in internal pH, until they reached equilibrium with the external pH. This was not due to a decrease in

Table 1. *Comparison of measurements of volume and fluid transport rates by diameter measurements and by using tritiated water as a tracer*

d(t0)	d(S)	V(t0)	d(t)	V(t)	Vt–Vt0	d.p.m.	d.p.m. (med)	$V(^3H_2O)$	r(calc)	$r(^3H_2O)$
(A) ^3H_2O administered after spheres sealed:										
533	845	79·0	650	143·6	64·6	826	141588	58·3	4·0	3·7
585	936	104·6	676	161·6	57·0	1476	180658	81·7	3·0	4·3
455	650	48·9	624	126·8	77·9	238	144257	16·5	6·8	1·4
364	559	25·2	468	53·6	28·4	243	178106	13·6	3·9	1·9
(B) ^3H_2O administered with explants unsealed:										
—	1560	—	975	161	—	1143	179689	63·6		
—	845	—	910	48·1	—	413	185152	22·3		
—	624	—	494	62·8	—	156	154733	10·1		
—	1040	—	676	161·6	—	1394	179427	77·7		

Each row represents measurements made on a single explant. The first four explants (A) were allowed to seal before the tracer was administered, whilst the second four (B) were placed in tritiated water whilst the explants were still unsealed. d(t0) = diameter of sphere at time when tritiated water added (μm); d(S) = diameter of sphere of equivalent surface area to a circle of diameter d(t0) calculated as described in the methods (μm); V(t0) = volume of sphere at the time when tritiated water was added (nl); d(t) = diameter of sphere at time t (7 h) after incubation in the presence of label (μm); V(t) = volume of this sphere (corrected for Form-factor) (nl); Vt-Vt0 = increase in volume between time t and time 0 (nl); d.p.m. = total disintegrations per minute counted after washing sphere as described in the methods; d.p.m. (med) = disintegrations per minute in a 10 μl sample of medium taken at the end of incubation period; $V(^3H_2O)$ = volume as determined from d.p.m. and d.p.m.(med) (nl); r(calc) = rate of water transport calculated from volumes measured by diameter of spheres ($\mu l\,cm^{-2}\,h^{-1}$); $r(^3H_2O)$ = rate of water transport calculated from tritiated water data ($\mu l\,cm^{-2}\,h^{-1}$). The surface areas of the four explants in group (A) were, respectively: 223123, 268783, 162597 and 104062 μm^2. In group (A), compare rates r(calc) and $r(^3H_2O)$ and increase in volumes Vt-Vt0 and $V(^3H_2O)$. In group (B), compare final volumes V(t) and $V(^3H_2O)$.

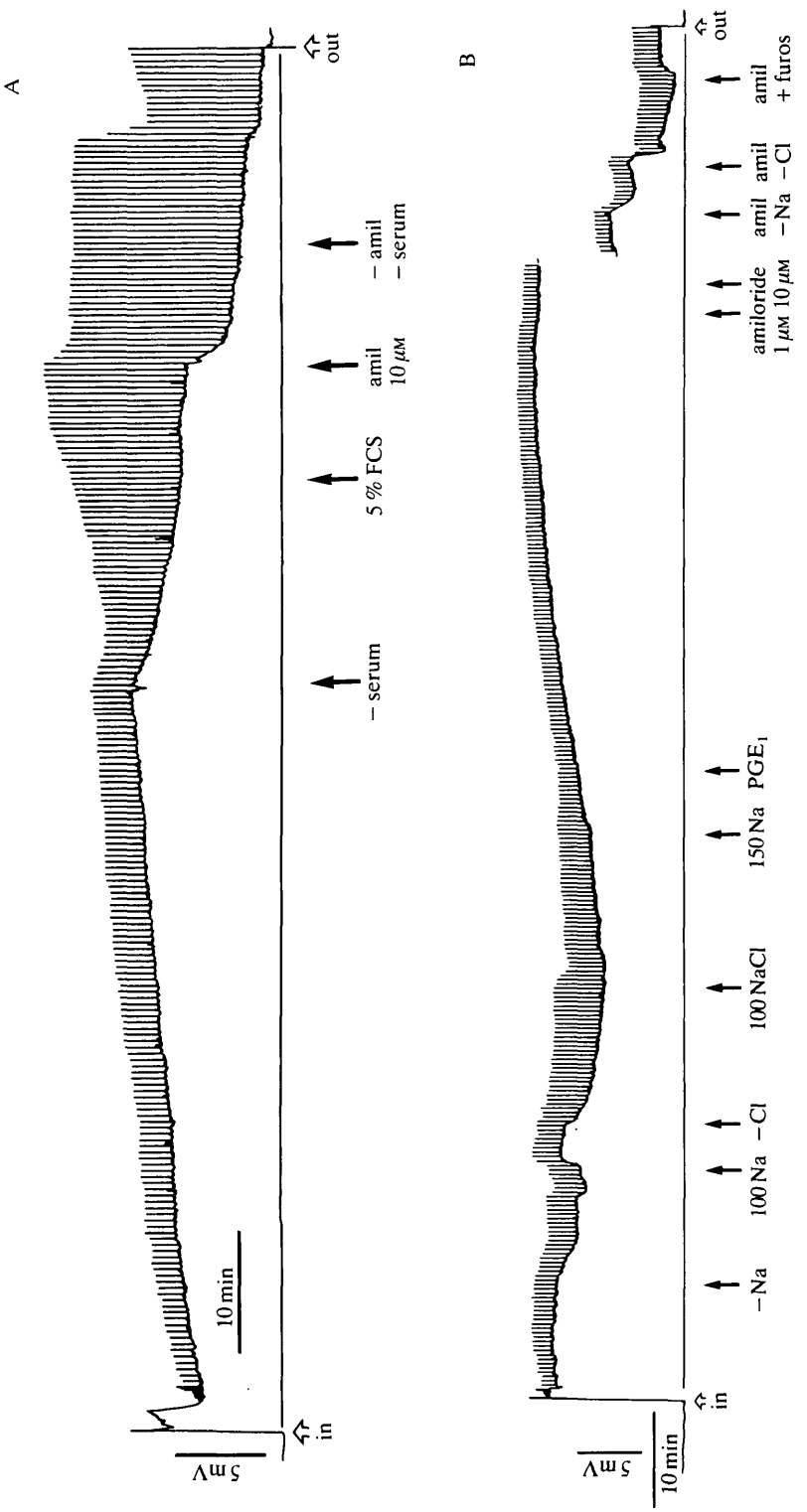

Fig. 4A,B. Electrophysiological records from two spheres each impaled with two 3 M-KCl microelectrodes. The heavy trace indicates the transepithelial potential, whilst the length of the individual spikes is proportional to the transepithelial resistance. The effects of various inhibitors administered to the bath are shown.

transepithelial resistance, since this concentration of amiloride increased the resistance (see above and Fig. 4).

Effect of external serum concentration on rate of fluid uptake

Explants placed in serum-free Pannett-Compton saline (n = 12) displayed an inability to seal, which was overcome by the addition of serum (0·5 %) (n = 5).

The effect of external foetal calf serum concentration on the rate of swelling of the spheres was investigated. The results are shown in Fig. 5. The figure shows a sharp peak ($4{\cdot}5 \pm 2{\cdot}0\,\mu l\,cm^{-2}\,h^{-1}$, n = 13) in the rate of fluid uptake at a serum concentration of 5 % in medium 199. Explants placed in serum-free medium 199 (n = 6) sealed up normally but failed to undergo any measurable volume increase. 0·5 % serum was sufficient to allow some fluid transport (n = 6).

Experiments with inhibitors and Na, Ca, K and Cl depletion

The effects of various inhibitors of sodium transport, of PGE1, of a calcium channel blocker, of inhibitors of vesicular traffic and of media of different ionic composition on the rate of fluid uptake were investigated, and the results are summarized in Table 2.

Strophanthidin is a cardiac glycoside which acts as an inhibitor of the Na,K-ATPase or sodium pump, which is located towards the basal or inner aspect of the explants (Stern & MacKenzie, 1983). The drug had no effect on the rate of fluid uptake in concentrations up to 10^{-5}M, if added after the spheres had sealed and started to swell. On the other hand, 10^{-5}M-strophanthidin reduced the rate of

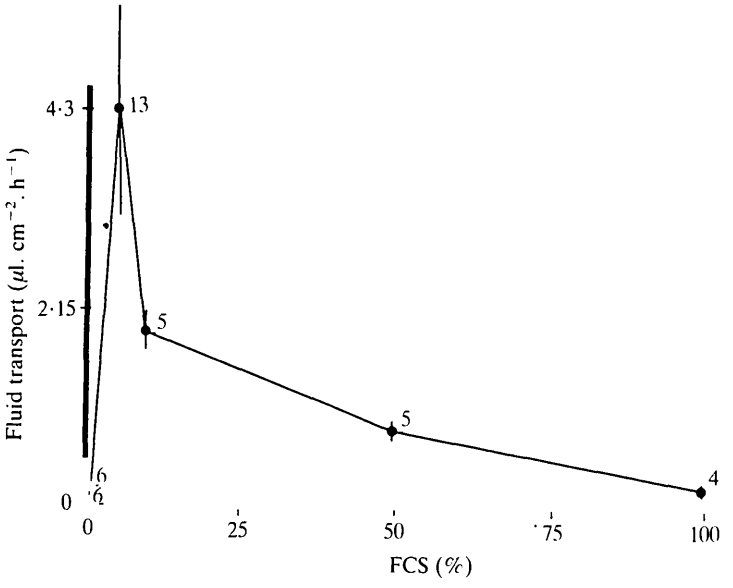

Fig. 5. Foetal calf serum concentration-dependence of the rate of fluid transport into the spheres.

swelling to $0{\cdot}2 \pm 0{\cdot}07\ \mu l\ cm^{-2}\ h^{-1}$ ($n = 11$; $P < 0{\cdot}0001$ with respect to controls) if added at the beginning of the experiment so that the basal aspect of the sheet was exposed to the inhibitor.

Furosemide and amiloride are blockers of two different 'slow' sodium channels, thought to reside at the apical (outer) surfaces of epithelial cells. Furosemide is probably an inhibitor of sodium/chloride influx symports (Sullivan, Tucker & Scherbenske, 1971; Montoreano, Rabito & Villamil, 1975). At 5×10^{-4}M administered after the explants had sealed, furosemide slightly reduced the transport rate to $2{\cdot}4 \pm 1{\cdot}2$ (S.D.) $\mu l\ cm^{-2}\ h^{-1}$ ($n = 5$; Not significantly different from controls: $P = 0{\cdot}168$). Amiloride is an inhibitor of the serum-stimulated component of sodium influx, where sodium is believed to enter the cells in exchange for hydrogen ions (see Smith & Rozengurt, 1978; Reznik, Villela & Mendoza, 1983). Amiloride (10 μM) reduced the transport rate to $1{\cdot}0 \pm 0{\cdot}7\ \mu l\ cm^{-2}\ h^{-1}$ ($n = 7$; $P < 0{\cdot}01$). Amiloride and furosemide administered together further reduced the transport rate to $0{\cdot}133 \pm 0{\cdot}159\ \mu l\ cm^{-2}\ h^{-1}$ ($n = 6$, $P < 0{\cdot}0001$). 1 μM-Tetrodotoxin (TTX), which is an inhibitor of 'fast' sodium influx in excitable tissues, had no

Table 2. *Summary of the effects of different inhibitors and media of various compositions on the rate of water flux and on transepithelial potential*

Treatment	Effect on transport	Effect on potential
Control (5 % FCS)	$4{\cdot}5 \pm 2{\cdot}0\ \mu l\ cm^{-2}\ h^{-1}$	(+6mV)
Na^+-free	$1{\cdot}9 \pm 0{\cdot}4$*	−3mV
K^+-free	$0{\cdot}8 \pm 0{\cdot}3$†	
Cl^--free	No effect	−3mV
Ca^{2+}-free	0	
pH 6·0	No effect	(inside pH 6·8 → 6·4)
pH 9·0	No effect	(inside pH 6·8 → 8·8)
10 μM-strophanthidin:		
outside:	No effect	No effect
both sides:	$0{\cdot}2 \pm 0{\cdot}07$‡	
1 μM-amiloride	No effect	
10 μM-amiloride	$1{\cdot}0 \pm 0{\cdot}7$†	−2mV
500 μM-furosemide	$(2{\cdot}4 \pm 1{\cdot}2)$	No effect
amil + furosemide (10; 500 μM)	$0{\cdot}1 \pm 0{\cdot}16$‡	−3mV
1 μM-TTX	No effect	
1 μM-nifedipine	$1{\cdot}2 + 0{\cdot}6$†	
10 μM-nifedipine	$1{\cdot}1 \pm 0{\cdot}5$†	
0·1 μM-monensin	No effect	
1 μM-monensin	0 (irrev.)	
1 μM-cytochal.-B	0	
1 mM-dBcAMP	$1{\cdot}3 \pm 0{\cdot}5$†	
1 μM-PGE 1	$0{\cdot}9 \pm 0{\cdot}2$‡	+5 mV

'0' = complete inhibition. Effect on transport is expressed as the rate of fluid increase measured in the presence of the inhibitor in $\mu l\ cm^{-2}\ h^{-1}$. *, $P < 0{\cdot}05$; †, $P < 0{\cdot}01$; ‡, $P < 0{\cdot}0001$. Effect on potential is expressed as the change observed in the presence of the inhibitor with respect to controls (5 % foetal calf serum) in mV. A minus sign indicates a decrease in transepithelial potential, and a plus sign an increase in potential by amount shown.

detectable effect on fluid uptake, which supports the notion that these channels are absent from non-excitable cells.

In sodium-free (choline substituted) medium the explants did not seal into spheres. When explants were already folded at the time when they were placed in sodium-free medium (n = 10), they began to unfold, taking about 10 h to do so. The sheets remained intact, but when placed back into 120 mM-sodium they totally disintegrated within 15 h. When explants were allowed to seal in normal (5 % foetal calf serum/199) medium and were then transferred to sodium-free medium, the spheres did increase in volume, but the rate of fluid uptake was reduced to $1{\cdot}9 \pm 0{\cdot}4\,\mu l\,cm^{-2}h^{-1}$ (n = 3; $P < 0{\cdot}05$). When these spheres were returned to normal medium, the rate of swelling increased to $3{\cdot}0 \pm 0{\cdot}02\,\mu l\,cm^{-2}h^{-1}$ (n = 4; Not significantly different from controls). Halving the sodium concentration to about 60 mM had no effect on the rate of swelling.

1 μM-PGE1, which increased the transepithelial potential, decreased the rate of fluid transport into the spheres to $0{\cdot}9 \pm 0{\cdot}2\,\mu l\,cm^{-2}h^{-1}$ (n = 6; $P < 0{\cdot}0001$). 1 mM-Dibutyryl cAMP, which has been reported to inhibit fluid transport in retinal pigmented epithelium (Miller, Hughes & Machen, 1982) was also found to be inhibitory ($1{\cdot}3 \pm 0{\cdot}5\,\mu l\,cm^{-2}h^{-1}$, n = 6; $P < 0{\cdot}01$) in the present experiments.

In potassium-depleted medium, the spheres sealed up normally but the rate of fluid uptake was reduced to $0{\cdot}8 \pm 0{\cdot}3\,\mu l\,cm^{-2}h^{-1}$ (n = 5; $P < 0{\cdot}01$). In chloride-free medium, the swelling of the spheres was not inhibited.

The possible rôle of calcium in the regulation of fluid uptake into the spheres was investigated by raising them in the presence of 1 or 10 μM-nifedipine, a calcium channel blocker. The inhibitor was found to decrease the rate of fluid uptake to $1{\cdot}2 \pm 0{\cdot}6\,\mu l\,cm^{-2}h^{-1}$ at 1 μM (n = 6; $P < 0{\cdot}01$) and to $1{\cdot}1 \pm 0{\cdot}5\,\mu l\,cm^{-2}h^{-1}$ at 10 μM (n = 7; $P < 0{\cdot}01$). Calcium-free medium caused the spheres (n = 12) to lose their integrity, and to rapidly deflate. This change was fully reversible when the spheres were placed back into normal medium.

An increase in external pH to 9–9·5 had no effect on the rate of fluid uptake (n = 6). Similarly, a reduction of pH to 6 had no significant effect (n = 5).

The possibility that fluid might be actively secreted by the tissue was investigated using the ionophore monensin and the microfilament inhibitor, cytochalasin-B. The former substance has been reported to act as an inhibitor of vesicular transport at low (i.e. $< 0{\cdot}1$–1 μM) concentrations (Tartakoff, 1983; Tartakoff & Vassalli, 1978) and as a sodium ionophore at higher (i.e. > 25 μM) concentrations (Sandeaux, Sandeaux, Gavach & Brun, 1982). In our hands, 10^{-7} M-monensin had little or no effect on the rate of swelling of the spheres, whilst at 10^{-6} M it inhibited transport of fluid in about half the specimens (19/42). However, inhibited specimens did not recover if transferred back into medium in the absence of the inhibitor. Higher concentrations, up to 50 μM, were also toxic. Cytochalasin-B (1 μM) rapidly and completely inhibited fluid uptake and caused the spheres to deflate. Returning them to medium in the absence of the inhibitor resulted in a return to control rates of fluid uptake.

DISCUSSION

(1) *Evaluation of the technique for quantifying transport rates*

Romanoff (1943*a*,*b*), Romanoff & Hayward (1943) and Adolph (1967) estimated that the rate of water flow into the sub-blastodermic cavity of the chick is of the order of $1{\cdot}8\,\mu l\,cm^{-2}\,h^{-1}$, based on measurements of the volume of this cavity. This figure agrees well with the flux determinations in the present study.

A comparison between volumes and flux rates estimated by the present 'volumetric' method and by the more conventional ^3H_2O-uptake determination (Table 1) showed that although both methods yield results of a similar order, there is a more-or-less consistent discrepancy (which can amount to 50 %) between them. Our impression is that both results reflect errors. The volumetric method tends to overestimate owing to factors such as optical distortion, and to the thickness of the tissue, which cannot be determined. The tracer method, on the other hand, tends to underestimate because some of the label is lost during the washing procedure. An attempt to determine the efflux of label during the washes was unsuccessful because of the low level of activity detectable in the wash solutions. The reason for this is that the spheres have a volume of only a few nl: even with the highest level of labelling which we have used, each sphere contained a total of no more than 1500 d.p.m. when the incubation medium contained $1{\cdot}8\times10^5$ d.p.m. $10\,\mu l^{-1}$. Small fractions of the radioactivity within the sphere which are lost to the wash solutions, therefore, cannot be accurately determined under these conditions.

Because of the ease with which volume and rate measurements may be made by the volumetric method, this was the method of choice in our experiments. It has the added advantage that by using time-lapse filming the rate of fluid uptake can be monitored continuously, even through a change of solution.

(2) *General behaviour of excised epiblast*

(i) The readiness with which the excised epiblasts fold in the manner described and seal along their edges suggests more than a random response to exceptional circumstances. New (1956) observed that under certain conditions whole embryos fold with their basal surface inwards. The direction of folding may be dependent on the position of cytoskeletal components. We have found that sheets of epiblast explanted directly into cytochalasin-B did not fold in this way.

(ii) The ability of the spheres to undergo a large increase in volume suggests that the epiblast is capable of net unidirectional fluid transport and that it can resist large hydrostatic pressure.

The transporting properties of the tissue confirm the observations of New (1956), of Elias (1964) and of Steding & Christ (1969) that the early chick embryo transports fluid from its dorsal (albumen-facing) side to the yolk-facing side, leading to the formation of the sub-blastodermic fluid.

The extrusion of material during the rapid deflationary phase is evidence of this high internal pressure. Evidently, the sphere is sufficiently tight to withstand the large accumulation of fluid, and it seems that the seal formed between the edges of the sheet is as tight as the rest of the epiblast. Bellairs, Breathnach & Gross (1975) have already shown that in the early chick embryo (stages 1–3) intercellular tight junctions are truly tight.

(iii) The behaviour of the explants containing a primitive streak, and in particular the observation that the majority never ruptured, is indicative of an area of leakage which limits the volume increase. This proposition was originally suggested on theoretical grounds (Stern, 1984), and the present observations give support to the concept that the primitive streak represents a region the function of which is to relieve the electrochemical disequilibrium across the epiblast in the chick embryo. Ouabain-binding studies have shown that the localization of sodium pumps is at the apical side of the primitive streak, in contrast to the rest of the epiblast (Stern & MacKenzie, 1983). The pattern of current flow at the primitive streak is also reversed with respect to other areas (Jaffe & Stern, 1979). These observations strongly suggest that sodium is transported out of the embryo at the primitive streak.

(3) *The nature of the driving forces for fluid transport*

Fundamental to the implication of sodium in fluid transport is that both should be transported in the same direction, water following the osmotic gradient produced by the movement of sodium. Our present experiments indicate that chloride is most likely to be the counterion, because of the sensitivity of the transepithelial potential to chloride depletion and to treatment with furosemide. Net fluid transport in the spheres is from the apical surface to the basal surface. The direction is the same as that which sodium follows in its unidirectional transport across the epiblast (Howard, 1957; Stern & MacKenzie, 1983).

That these spheres do transport sodium into their interior as do sheets of the same tissue in 'mini-Ussing' chambers (Stern & MacKenzie, 1983) is indicated by the presence of a transepithelial potential (inside of sphere positive) and by the sensitivity of this potential to inhibitors of sodium entry into the cells (amiloride, furosemide, sodium-free medium).

The connection between unidirectional sodium transport and unidirectional fluid transport is evident from the observation that sodium transport inhibitors (amiloride with or without furosemide, strophanthidin and sodium-free medium) all markedly reduce the rate of fluid uptake into the spheres.

Amiloride can totally inhibit the serum-stimulated component of sodium influx whilst inhibiting less than 10 % of the sodium influx in serum-deprived cells (Smith & Rozengurt, 1978; see also Reznik *et al.* 1983). The component of sodium influx which is not serum dependent may be facilitated by, or coupled with transport of chloride and/or phosphate, as is found in the toad bladder (Chen & Walser, 1974). Furosemide has been shown to inhibit both active chloride influx and passive chloride efflux (Montoreano *et al.* 1975) and in this way it may indirectly reduce the

serum-independent sodium transport. Furosemide may also have a direct effect on the sodium transport mechanism (Sullivan *et al.* 1971). So, if combined with amiloride, furosemide should further reduce the fluid uptake by the spheres. The observations in the present study confirm these suggestions. Serum has a stimulatory effect on both the transepithelial potential and on the rate of fluid uptake by the spheres. Furosemide alone has a small inhibitory effect on the rate of fluid transport, amiloride alone reduces the uptake of fluid to a level similar to that in very low levels (0·5 %) of serum. Amiloride and furosemide combined further inhibit fluid uptake to a level similar to that in the absence of sodium.

These observations suggest the co-existence of more than one pathway for sodium entry into the cells. We suggest that there are at least two separate sodium channels at the apical side of the tissue: (1) a serum-stimulated, amiloride sensitive channel, where sodium may be transported into the cell in exchange for protons, (2) a furosemide-sensitive, chloride-dependent channel, where sodium may enter the cell together with chloride ions. Efflux of sodium from the cells, on the other hand, takes place at the basolateral side of the tissue, via Na,K-ATPase pumps, since *in ovo* all of the transepithelial potential can be inhibited with strophanthidin. Unfortunately we were unable to confirm this observation electrophysiologically using spheres, since the basal aspect of the sheets is not exposed to the external medium. Apically administered strophanthidin had no effect on the transepithelial potential, and injection of strophanthidin into the spheres presents considerable technical difficulties.

Sodium does not appear to be the only factor responsible for generating the driving force for unidirectional fluid transport into the spheres, since none of the sodium transport inhibitors used here (including strophanthidin offered to the basal aspect of the sheet, which completely inhibited the transepithelial potential, Stern & MacKenzie, 1983) were capable of completely eliminating fluid uptake. Furthermore, New (1956) and House (1974) argued on the basis of the experiments of the former author that the blastoderm is capable of fluid transport in the absence of any osmotic gradients.

The results indicate that the rate of fluid uptake is independent of the transepithelial pH gradient, and relatively independent of the transepithelial potential since PGE1 which increased the latter had an *inhibitory* effect on the rate of fluid transport. In frog skin, PGE1 was found to increase the short-circuit current and to decrease transepithelial resistance (Hall & O'Reagan, 1975). Our results support this finding in that PGE1 increased the transepithelial potential whilst decreasing transepithelial resistance. Lipson, Hynie & Sharp (1971) and Lipson & Sharp (1971), demonstrated an antagonist effect of the prostaglandin on water flux and a stimulatory effect of the drug on sodium transport in the toad bladder. They argued that this provides evidence for the existence of two distinct adenyl cyclase mechanisms, one regulating sodium transport and the other controlling water flux. The mode of action of PGE1 on water flux appears to be through an inhibition, probably mediated by cAMP, of the osmotic transepithelial permeability in response to basal hypertonicity (Ripoche, Bourguet & Parisi,

1973). Our results appear to corroborate these conclusions for the early chick embryo. The possible involvement of cAMP in fluid transport was further investigated by adding 1 mM-dibutyryl cAMP as Miller *et al.* (1982) have done, and it was indeed found to slightly inhibit fluid uptake into the spheres.

Calcium may be implicated in the residual water transport, either in connection with the cAMP pathway mentioned above, or with vesicular transport (through its effects on exocytosis and endocytosis, Baker & Knight, 1981, or on microfilament function and therefore cytoplasmic movements, see Stern, 1984). The proposition that calcium homeostasis is involved in some way in fluid transport receives support from the observation in the present study that nifedipine can inhibit some of the transport of fluid into the spheres. Calcium-free experiments, however, cannot give information about the driving force for fluid, since this medium interferes with the integrity of the epithelial sheets.

Two further possibilities remain for the 'residual' transport. The first is that fluid is transported in vesicles which traverse the cell from the apical to the basal side. The second possibility is the suggestion of Tuft (1962, 1965) that there is 'active' water transport by the blastoderm. Both propositions are difficult to test experimentally.

Mechanisms relying on vesicular forms of transport have once been condemned as 'the last refuge of the intellectually bankrupt' by Hogben (1960). Although there have been numerous studies of both exocytosis and endocytosis in a variety of systems (e.g. for the chick embryo, MacLean & Sanders, 1983), there is little evidence for the involvement of vesicles in transcellular transport, apart from a few studies (Palade, 1953, 1982; Cohn & Steinman, 1982). Cohn & Steinman (1982) quantified rates of pinocytosis and phagocytosis and their figures for the rate of influx of macrophage pinocytic vesicles ($102\,\mu m^3\,cell^{-1}\,h^{-1}$, which is equivalent to $0{\cdot}2\,\mu l\,cm^{-2}\,h^{-1}$ for the chick epiblast assuming a cell density of $1{\cdot}78\times10^6$ cells cm^{-2}; Stern & MacKenzie, 1983) are similar to the rates of trans-epithelial fluid transport we have observed in the presence of strophanthidin. Nevertheless, New (1956) and House (1974) argued that *normal* rates of fluid transport can take place in the absence of osmotic gradients. These considerations suggest that vesicular transport could indeed account for the whole of the residual transport in the absence of sodium transport. Our attempts to inhibit a possible vesicular pathway for fluid transport using monensin or cytochalasin-B are inconclusive because of the non-specific, toxic effects of both substances. Cytochalasin-B appears to inhibit transport mainly because it disrupts the integrity of the tissue, so that no fluid can accumulate. The effect of monensin on water flux has also been investigated by Mendoza & Thomas (1982), who found no effect of the ionophore on water fluxes but that it inhibited the stimulatory effects of vasopressin and of cyclic AMP in the toad bladder. In our own experiments, however, cyclic AMP had an *inhibitory* effect on water transport.

Finally, there is Tuft's (1962, 1965) suggestion of active secretion of fluid. Jurand & Tuft (1961) argued from their experiments using beta-mercaptoethanol that there is indeed 'active' fluid secretion by the blastoderm. In the present study we

have been unable to design an experiment which would directly test this possibility. Metabolic inhibitors such as dinitrophenol are far too non-specific in their effect to be useful for this purpose.

We must therefore conclude that sodium transport is responsible for most (about 90%) of the fluid transport by the epiblast. The residual, non-sodium driven, transport, could be due to (a) a cAMP-coupled transport pathway, (b) active secretion, (c) a calcium-entry-requiring mechanism, which could be the same mechanism as (d) vesicular transport, or a combination of these factors. It is at present impossible to decide between them.

The work of one of us (S.M.) was done as part of a B.Sc. Honours project at University College London under the supervision of C.D.S. C.D.S. was supported by a grant from the Science and Engineering Research Council to Professor Ruth Bellairs, to whom we are grateful for her support and for the use of facilities. We are also grateful to Dr A. E. Warner for her helpful comments and advice and to Mrs Rosalyn Cleevely for technical assistance.

REFERENCES

ADOLPH, E. F. (1967). Ontogeny of volume regulations in embryonic extracellular fluids. *Q. Rev. Biol.* **42**, 1–39.

BAKER, P. F. & KNIGHT, D. E. (1981). Calcium control of exocytosis and endocytosis in bovine adrenal medullary cells. *Phil. Trans. Roy. Soc. Lond. B.* **296**, 83–103.

BELLAIRS, R. (1971). *Developmental Processes in Higher Vertebrates.* London: Logos Press.

BELLAIRS, R., BREATHNACH, A. S. & GROSS, M. (1975). Freeze-fracture replication of junctional complexes in unincubated and incubated chick embryos. *Cell Tiss. Res.* **162**, 235–252.

BELLAIRS, R., IRELAND, G. W., SANDERS, E. J. & STERN, C. D. (1981). The behaviour of embryonic chick and quail tissues in culture. *J. Embryol. exp. Morph.* **61**, 15–33.

BELLAIRS, R., SANDERS, E. J. & PORTCH, P. A. (1978). In vitro studies on the development of neural and ectodermal cells from young chick embryos. *Zoon* **6**, 36–50.

BORLAND, R. M., BIGGERS, J. D. & LECHENE, C. P. (1976). Kinetic aspects of rabbit blastocoele fluid accumulation. An application of electron probe microanalysis. *Devl Biol.* **50**, 201–211.

BORLAND, R. M., BIGGERS, J. D. & LECHENE, C. P. (1977). Studies on the composition and formation of mouse blastocoele fluid using electron probe microanalysis. *Devl Biol.* **55**, 1–8.

BRECKENRIDGE, L. J. & WARNER, A. E. (1982). Intracellular sodium and the differentiation of amphibian embryonic neurones. *J. Physiol.* **332**, 393–413.

CHEN, J. S. & WALSER, M. (1974). Passive ion fluxes across toad bladder. *J. membr. Biol.* **18**, 365–378.

COHN, Z. A. & STEINMAN, R. M. (1982). Phagocytosis and fluid-phase pinocytosis. In *Membrane Recycling* (ed. Evered & Collins), pp. 15–34. London: Pitman Books (Ciba Fdn. Symp. 92).

CROSS, M. H. (1973). Active sodium and chloride transport across the rabbit blastocoele wall. *Biol. Reprod.* **8**, 566–575.

DAVY, J. (1863). Some observations on the eggs of birds. *Brit. Assoc. Rep.* 1863 (2), 112.

DEHAAN, R. L. (1960). Avian embryo culture. In *Methods in Developmental Biology* (ed. F. H. Wilt & N. K. Wessels), pp. 401–412. New York: Thomas Crowell.

ELIAS, S. (1964). The subembryonic liquid in the hen's egg (Formation and biochemistry). *Rev. roumaine. Embryol. Cytol.* **1**, 165–192.

EYAL-GILADI, H. & KOCHAV, S. (1976). From cleavage to primitive streak formation: a complementary normal table and a new look at the development of the chick embryo. *Devl Biol.* **49**, 321–337.

HALL, W. J. & O'REAGAN, M. G. (1975). Effect of prostaglandin E1 and other factors on the electrical excitability of isolated frog skin (*Rana temporaria*). *Gen. Pharmacol.* **6**, 151–154.

HAMBURGER, V. & HAMILTON, H. L. (1951). A series of normal stages in the development of the chick. *J. Morph.* **88**, 49–92.

HOGBEN, C. A. M. (1960). Movement of material across cell membranes. *Physiologist* **3**, 56–62.

HOUSE, C. R. (1974). *Water Transport in Cells and Tissues*. London: Edward Arnold.

HOWARD, E. (1957). Ontogenetic changes in the freezing point and sodium and potassium content of the subgerminal fluid and blood plasma of the chick embryo. *J. cell. comp. Physiol.* **50**, 451–470.

JAFFE, L. F. & STERN, C. D. (1979). Strong electrical currents leave the primitive streak of chick embryos. *Science* **206**, 569–571.

JURAND, A. & TUFT, P. (1961). Distribution of water in the chick blastoderm. *Nature* **191**, 1073–1074.

LIPSON, L., HYNIE, S. & SHARP, G. (1971). Effect of prostaglandin E1 on osmotic water flow and sodium transport in the toad bladder. *Ann. N.Y. Acad. Sci.* **180**, 261–277.

LIPSON, L. C. & SHARP, G. W. G. (1971). Effect of prostaglandin E1 on sodium transport and osmotic water flow in the toad bladder. *Amer. J. Physiol.* **220**, 1046–1052.

MACLEAN, I. M. & SANDERS, E. J. (1983). Cationized ferritin and phosvitin uptake by coated vesicles of the early chick embryo. *Anat. Embryol.* **166**, 385–397.

MENDOZA, S. A. & THOMAS, M. W. (1982). Effect of monensin on osmotic water flow across the toad bladder and its stimulation by vasopressin and cyclic AMP. *J. memb. Biol.* **67**, 99–102.

MESSENGER, E. A. & WARNER, A. E. (1979). The function of the sodium pump during differentiation of amphibian embryonic neurones. *J. Physiol.* **292**, 85–105.

MILLER, S. S., HUGHES, B. A. & MACHEN, T. E. (1982). Fluid transport across retinal pigmented epithelium is inhibited by cyclic AMP. *Proc. natn. Acad. Sci., U.S.A.* **79**, 2111–2115.

MONTOREANO, R., RABITO, C. A. & VILLAMIL, M. F. (1975). Effect of furosemide on unidirectional fluxes of sodium and chloride across the skin of the frog, *Rana pipiens*. *Biochim. biophys. Acta* **455**, 831–836.

MURRAY, P. D. F. (1933). The cultivation in saline and other media of the haematopoietic region of the early chick embryo. *Arch. exp. Zellforsch.* **14**, 574–594.

NEW, D. A. T. (1956). The formation of sub-blastodermic fluid in hens' eggs. *J. Embryol. exp. Morph.* **4**, 221–227.

PALADE, G. E. (1953). Fine structure of blood capillaries. *J. appl. Physics* **24**, 1424.

PALADE, G. E. (1982). Problems in intracellular membrane traffic. In *Membrane Recycling* (ed. Evered & Collins), pp. 1–14. London: Pitman Books (Ciba Fdn. Symp. 92).

PURVES, R. D. (1981). *Microelectrode Methods for Intracellular Recording and Ionophoresis*. London: Academic Press.

REZNIK, V. M., VILLELA, J. & MENDOZA, S. A. (1983). Serum stimulates Na entry and the Na-K pump in quiescent cultures of epithelial cells (MDCK). *J. cell. Physiol.* **117**, 211–214.

RIPOCHE, P., BOURGUET, J. & PARISI, M. (1973). The effect of hypertonic media on water permeability of frog urinary bladder. Inhibition by catecholamines and prostaglandin E1. *J. gen. Physiol.* **61**, 110–124.

ROMANOFF, A. L. (1943*a*). Assimilation of avian yolk and albumen under normal and extreme incubating temperatures. *Anat. Rec.* **86**, 143–148.

ROMANOFF, A. L. (1943*b*). Distribution of dry constituents of yolk and albumen in the developing avian egg. *Anat. Rec.* **87**, 303–306.

ROMANOFF, A. L. (1967). *Biochemistry of the Avian Embryo*. New York: Wiley.

ROMANOFF, A. L. & HAYWARD, F. W. (1943). Changes in volume and physical properties of allantoic and amniotic fluids under normal and extreme temperatures. *Biol. Bull. mar. biol. Lab., Woods Hole* **84**, 141–147.

ROMANOFF, A. L. & ROMANOFF, A. J. (1949). *The Avian Egg*. New York: Wiley.

SANDEAUX, R., SANDEAUX, J., GAVACH, C. & BRUN, B. (1982). Transport of sodium by monensin across bimolecular lipid membranes. *Biochim. biophys. Acta* **684**, 127–132.

SANDERS, E. J. & DICKAU, J. E. (1981). Morphological differentiation of an embryonic epithelium in culture. *Cell Tiss. Res.* **220**, 539–548.

SCHULTHESS, P., SHIJO, Y., PHAM, H. V., PRETSCH, E., AMMANN, D. & SIMON, W. (1981). A hydrogen ion-selective liquid-membrane electrode based on tri-n-dodecylamine as neutral carrier. *Analytica chim. Acta* **131**, 111–116.

SHARP, P. F. & POWELL, C. K. (1931). Increase in pH of the white and yolk of hens' eggs. *Industr. Eng. Chem.* **23**, 196–199.

SHKLYAR, N. (1937). A study of physico-chemical changes in the egg during embryonic development in birds. I. Changes in the concentration of hydrogen ions in relation to embryonic development in the eggs of domestic fowl (hens, turkeys, ducks, geese). *Ukr. Biochem. J.* **10**, 405–406.

SMITH, J. B. & ROZENGURT, E. (1978). Serum stimulates the Na/K pump in quiescent fibroblasts by increasing Na entry. *Proc. natn. Acad. Sci., U.S.A.* **75**, 5560–5564.

SPRATT, N. T. (1947). Development in vitro of the early chick blastoderm explanted on yolk and albumen extract saline-agar substrates. *J. exp. Zool.* **106**, 345–365.

STEDING, G. & CHRIST, B. (1969). Die Bedeutung des Wassertransportes im Stoffwechselfeld des Ektoderm. *Anat. Anz. Erganzungs.-H.* **125**, 785–790.

STERN, C. D. (1984). A simple model for early morphogenesis. *J. theor. Biol.* **107**, 229–242.

STERN, C. D. & IRELAND, G. W. (1981). An integrated experimental study of endoderm formation in avian embryos. *Anat. Embryol.* **163**, 245–263.

STERN, C. D. & MACKENZIE, D. O. (1983). Sodium transport and the control of epiblast polarity in the early chick embryo. *J. Embryol. exp. Morph.* **77**, 73–98.

SULLIVAN, L. P., TUCKER, J. M. & SCHERBENSKE, M. J. (1971). Effect of furosemide on sodium transport and metabolism in the toad bladder. *Amer. J. Physiol.* **220**, 1316–1324.

TARTAKOFF, A. M. (1983). Perturbation of vesicular traffic with the carboxylic ionophore monensin. *Cell* **32**, 1026–1028.

TARTAKOFF, A. M. & VASSALLI, P. (1978). Comparative studies of intracellular transport of secretory proteins. *J. Cell Biol.* **79**, 694–707.

THOMAS, R. C. (1978). Ion-sensitive Intracellular Microelectrodes. London: Academic Press.

TUFT, P. H. (1962). The uptake and distribution of water in the embryo of *Xenopus laevis* (Daudin). *J. exp. Biol.* **39**, 1–19.

TUFT, P. H. (1965). The uptake and distribution of water in the developing amphibian embryo. *Symp. Soc. exp. Biol.* **19**, 385–402.

TUFT, P. H. & BOVING, B. G. (1970). The forces involved in water uptake by the rabbit blastocyst. *J. exp. Zool.* **174**, 165–172.

WLADIMIROFF, G. E. (1926). Beiträge zur Embryochimie und Embryophysiologie. I. Mitteilung: Einige physikalisch-chemische Veränderungen des Eierweisses sich entwickelnder Hühnereier. *Biochem. Z.* **177**, 280–297.

YAMADA, K. (1933). Über die Verteilung von Chlor in sich entwickelnder Hühnereiern. *Jap. J. Med. Sci. II. Biochemistry* **2**, 7179.

(Accepted 14 February 1985)