

Sodium transport and the control of epiblast polarity in the early chick embryo

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SUMMARY

The sodium transport properties of chick epiblast during gastrulation were studied by various techniques. It was found that the epiblast is capable of unidirectional apical to basal sodium transport, in towards the underlying intraembryonic space. The Na-K-ATPase was localized by [³H]ouabain binding and autoradiography near the basal surfaces of the cells, and the number of pump sites was quantified. The transport rate of sodium was determined with ²²Na. Electrophysiological studies on embryos at primitive streak stages showed a trans-epithelial potential of about $+16 \pm 5$ mV (basal side positive) which was sensitive to strophanthidin. Applying similar voltages but of reverse polarity to isolated sheets of epiblast caused a rapid reversal of some of their morphological polarity markers as well as some of their physiological functions. The relevance of these results to development is discussed.

INTRODUCTION

During the early stages of its development the chick embryo (Bellairs, 1971, 1981; Nicolet, 1971 for reviews) gradually passes from a single germ layer to three. The epiblast or upper layer is the first to be present. It consists of cells arranged as a pseudostratified epithelium with the ultrastructural features of a transporting tissue (Ziegler, 1977): a basal lamina covering its basal side (Sanders, 1979; Vanroelen, Vakaet & Andries, 1980*a,b,c*; Wakely & England, 1979) and intercellular tight junctions and microvilli at the apical ends of the cells (Bancroft & Bellairs, 1974; Bellairs, Breathnach & Gross, 1975; Buck, Ohara & Daniels, 1976; Revel, Yip & Chang, 1973; Sanders, 1973; Stolinski, Sanders, Bellairs & Martin, 1981). Later the lower layer, or hypoblast, forms. This layer appears to play an important role in the induction of the primitive streak (Waddington, 1932). The streak itself is the focus for the formation of the third layer, the mesoderm, which is situated between the other two.

The developmental physiology of these early chick embryos has not as yet been extensively researched. Sheridan (1966, 1968) has found coupling between cells of the epiblast. More direct evidence for its transporting function comes

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from the studies of New (1956) and Elias (1964) who found evidence for water fluxes across the epiblast into the underlying spaces, and Howard's (1953, 1957) findings that sodium and potassium concentrations in the albumen and yolk of the early embryos are what would be expected if there was net transport of sodium into the yolk and of potassium out to the albumen. The embryos have also been subjected to rather crudely applied electrical currents (Gray, 1934; Sedar, 1956) and it was found that these had teratological consequences due to both the electrical stimulus itself (Sedar, 1956) and to heavy metals released by the electrodes (Gray, 1934). The first major aim of this paper is to attempt to confirm unambiguously, from a physiological point of view, that the epiblast of the early chick embryo is indeed a transporting epithelium.

Electrophysiological studies using the vibrating probe have shown (Jaffe & Stern, 1979) that strong extracellular electrical currents leave the primitive streak region of intact cultured embryos dorsally. The pattern of current flow is consistent with the notion of a pump operating in the epiblast, pumping cations into the interior of the blastoderm, which then leave the space via the primitive streak. It has already been shown (Stern, 1981) that the lateral components of the current (i.e. those parallel to the plane of the germ layers) are probably not responsible for the guidance of mesoderm out of the primitive streak at these stages as mesoderm cells cannot respond to voltage gradients in a directional manner. That lateral voltage gradients could be guiding epiblast movements into the primitive streak seems equally unlikely, as the cells there have an even smaller diameter laterally than mesoderm cells, which implies that the potential drop per cell will be even smaller than in the middle layer.

These observations have led us to examine the possible role which the voltage drop across the thickness of the epiblast might be playing during gastrulation. Our second major aim in this paper is therefore to examine the possible morphological and physiological effects of this voltage drop.

Some of the results in this paper have been published in preliminary or abstract form (MacKenzie, 1980; Stern, 1982*a,b*).

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 XI to stage 5. Whole embryos were cultured according to the technique of New (1955) on their own vitelline membranes on a pool of egg albumen. Epiblasts (stage XIV-3) were cultured in the 'mini-Ussing' type chambers shown in Fig. 1 after removing any adhering hypoblast and/or mesoderm.

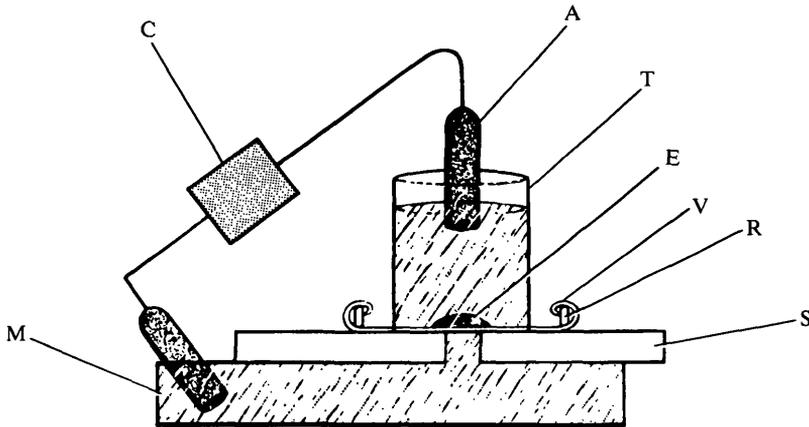


Fig. 1. Diagram of the experimental setup. The vitelline membrane (V) with the epiblast (E) attached was stretched around a glass ring (R) which was then waxed onto a glass microscope slide (S) with a 2.5 mm diameter hole cut out. A glass tube (T) was waxed into position inside the ring and provided with 1 to 2.5 ml medium (Pannett-Compton saline or medium 199 with or without 10 % foetal calf serum). In a few cases a millipore filter was placed between the membrane and the glass slide. The assembly was placed over a pool of medium in a shallow dish (M) and two Ag/AgCl probes (A) connected to the current source (C) were placed as shown. (Reproduced with permission from *Expl Cell Res.*)

For studies using inhibitors of the sodium pump embryos were cultured as previously described (New, 1955) on a pool of albumen containing the inhibitor in the desired concentration.

Autoradiography

Embryos were cultured by the New (1955) technique in a pool of albumen containing [^3H]ouabain at 2×10^{-7} M for 25 min (Simmons, 1981) applied to both sides of the vitelline membrane. Stock [^3H]ouabain (Radiochemical Centre, Amersham, U.K., specific activity 17 Ci/mmol) was administered as a total of 6 or 12 μCi per embryo. Embryos were then washed with 10 ml Pannett-Compton saline and either fixed in buffered formal saline, embedded in paraffin wax and sectioned in a histological microtome or sectioned in a cryostat after supporting in Tissutek embedding medium (Dow Corning). The sections were placed onto gelatinized slides (Rogers, 1979), covered with Ilford K2 emulsion, exposed with desiccant in the dark for up to 4 months and developed in Kodak D19b for up to 20 min. Control embryos were either offered the label in the presence of 15 mM- K^+ to test for non-specific binding, or were unlabelled to test for chemography artefacts.

Tritiated ouabain binding

Isolated epiblasts cultured in the mini-Ussing chambers as described above were exposed to 12 μCi [^3H]ouabain at 2×10^{-7} M, administered to either the

dorsal (apical) or the ventral (basal) side of the tissue. They were incubated in the presence of label for 25 min, washed thoroughly in three changes (10 ml each) of Pannett-Compton (1924) saline and then placed in scintillation vials. The tissue was digested using Protosol (New England Nuclear), neutralized with a few drops of glacial acetic acid to eliminate chemoluminescence artefacts and counted as a toluene-PPO-POPOP (BDH) mixture in a Beckmann LS7500 automatic scintillation counter. Errors due to quenching were controlled by the Beckmann Automatic Quench Correction (AQC) technique. The counting efficiency was between 17 and 50 %.

Transport of ^{22}Na

Radioactive ^{22}Na (isotonic solution, specific activity $25 \mu\text{Ci}/\text{mg Na}$, $100 \mu\text{Ci}/\text{ml}$, Amersham Radiochemical Centre) was administered as about $1 \mu\text{Ci}$ to either side of epiblasts cultured as described above. As the epiblasts could be cultured in the chambers either way up, any effects of gravity or hydrostatic pressure on the flux could be controlled. The label was therefore applied to random sides of the chamber. The tissues were cultured for about 5 h and $10 \mu\text{l}$ samples were withdrawn from the unlabelled side at 0, 10, 20, 30, 40, 50, 60, 90, 120, 150, 180, 240 and 300 min. In some experiments strophanthidin (Sigma) at 10^{-5}M was included in the medium on both sides of the tissue. At the end of the incubation period, the tissues were washed thoroughly with three 10 ml changes of saline, solubilized in Protosol, mixed with scintillation cocktail, neutralized with glacial acetic acid and counted as described above to determine the amount of sodium retained within the tissue. The aqueous samples were solubilized with 4 ml Triton X-100 (B.D.H.), mixed with 6 ml of the cocktail described above and counted in the scintillation counter by its positron emissions, with a counting efficiency between 86 and 90 %.

To determine the amount of sodium within the intra-embryonic space, embryos in New (1955) culture were grown in the presence of $0.5 \mu\text{Ci } ^{22}\text{Na}$ solution mixed with egg albumen for 30, 60, 120 and 180 min, washed quickly with three 10 ml changes of cold saline, digested with Protosol, mixed with scintillation fluid, neutralized with glacial acetic acid and counted as described. In some of the embryos the upper layer was separated from the lower layer to wash out the label from the cavity prior to counting. The amount of ^{22}Na in the cavity was determined as the difference between counts in washed embryos and counts in embryos which were dissected prior to washing.

In control experiments ^{22}Na solution was administered as described above to blastoderms which had been fixed in buffered formal saline overnight, or to vitelline membranes with no embryo attached. In both cases, no differences were found between sodium flux in either direction.

The results of flux measurements were 'normalized' by dividing the measured d.p.m. over the area of exposed tissue in the chamber. The results therefore represent d.p.m. per square mm.

Measurement of extracellular spaces

To estimate intercellular space in the epiblast, embryos (hypoblast dissected off) in New (1955) culture were exposed to $3 \mu\text{Ci}$ [^{14}C]mannitol (Amersham Radiochemical Centre, specific activity 59 mCi/mmol) in 1 ml medium (2:1 saline: egg albumen) and incubated for 2 h. They were then washed three times in 10 ml saline, digested with Protosol and counted as described above. A $10 \mu\text{l}$ sample of the incubation medium was also counted to adjust for any inaccuracy in the dispensing. The counting efficiency was between 93 and 95 %.

The volume of the blastodermic cavity was estimated by administering [^{14}C]mannitol to whole embryos which were then either washed or dissected and washed before counting as described for the ^{22}Na experiments above.

Estimation of cell number, DNA content and surface area

Cell number was estimated by counting cells per unit area in embryos (stage 3–5) stained using the silver technique of Arnolds (1979) (embryos kindly provided by Dr Arnolds to Prof. Bellairs) which highlights the contours of the cells. The surface area of epiblast exposed to the medium in the chambers was calculated by measuring the diameter of the hole in each chamber with a micrometer (0.01 mm resolution). DNA content was measured by a fluorimetric technique (Kissane & Robins, 1958).

Electrophysiology

For experiments done in the U.S.A., White Leghorn eggs were obtained from the Indiana Farm Bureau Co-op, and for those done in England Ross Rangers eggs were obtained from Ross Poultry (South) Ltd. The eggs were incubated at 38°C up to stage 4.

Electrophysiological studies *in ovo* (done mostly in the U.S.A.) were done by placing an egg (part of the shell removed) in a warmed, cushioned Plexiglass (Perspex) chamber, while the yolk was held down with a ring fixed to the end of a rod. Backfilled 3 M-KCl conventional microelectrodes (unbroken electrode resistances 10–50 M Ω) were referred to Ag/AgCl electrodes built into plexiglass holders (W. P. Instruments) filled with 3 M-KCl. Voltages were measured with a W. P. Instruments M-701 microprobe system electrometer (input resistance = $10^{11} \Omega$). Signals were displayed by a Tektronix 5A18N amplifier on a Tektronix D11 oscilloscope and on a Gould Brush 220 chart recorder. The preparation was referred to the system ground by means of a low-resistance Pannett-Compton saline bridge placed over the embryo, which was in turn referred to a 3 M-KCl solution and a W.P.I. Ag/AgCl electrode.

Studies on the effects of strophanthidin on the recorded potential (done in England) were performed *in ovo* using conventional 3 M-KCl microelectrodes

(resistance 2–10 M Ω) and a W.P.I. model FD223 electrometer (effective input resistance 10^{14} Ω) connected to 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, and were less than 1 mV. The temperature of the preparations was maintained at 37 °C by a hair dryer and a miniature bead thermistor probe in the egg, connected to a microprocessor (305-800)-controlled circuit. The albumen was replaced with Pannett-Compton saline. A stock solution of 10^{-3} M-strophanthidin (Sigma) in 10 % ethanol in saline was administered by injection into the yolk a few minutes after impalement to give a final concentration of 10^{-5} M in the whole egg. Egg volume had been estimated by water displacement.

For the application of standing voltages whole sheets of epiblast (dissected off any adhering mesoderm and endodermal layer cells) were cultured on their own vitelline membranes in the 'mini-Ussing' chambers shown in Fig. 1. The embryos could be mounted either way up. The setup was placed in a 37 °C incubator and currents between 6 and 100 μ A were applied to the Ag/AgCl electrodes (Clark Electromedical) by a simple constant current clamp device (Stern, 1981) for up to 6 h. The resistance across the sheets could be monitored by measuring the voltage with a WPI electrometer. Any polarization of the electrodes could thus be monitored. The current was then adjusted to give an initial voltage of 10–50 mV between the electrodes. In some experiments, two 3 M-KCl or Ag/AgCl probes were placed very close to both sides of the tissue to determine the actual voltage being applied across it. The difference between voltages at the electrodes and near the tissue never exceeded 5 mV. In long-term experiments (stimulation for 3 h or longer) the surface of the electrodes was cleaned with abrasive paper every 60 min to prevent changes in resistance due to buildup of electrode products. Applied voltages were never seen to increase significantly from the initial values.

Immunological techniques

An antibody against rabbit kidney Na/K-ATPase raised in rabbits was kindly provided by Dr E. J. J. vanZoelen (Hubrecht Laboratory, Utrecht, The Netherlands). Unfixed cryostat sections of chick embryos placed on gelatinized glass slides were air dried, washed with saline and then washed twice with a 0.1 M-lysine buffer made up in Pannett-Compton saline and again in the same saline to remove lysine. They were then incubated for 30 min at 37 °C in 60–80 μ l immune serum (1:10 in saline), washed again and finally incubated for another 30 min with either goat anti-rabbit IgG or goat anti-rabbit immunoglobulin coupled to fluorescein isothiocyanate (Nordic) (1:20 with saline). After thorough washing the sections were mounted in saline or 1:1 saline/glycerol and observed by epi-fluorescence microscopy. Controls were incubated either with normal rabbit serum or with heat-inactivated immune serum or with no rabbit serum. The rest of the procedure was unchanged.

Electron microscopy and histochemistry

Specimens for transmission electron microscopy (TEM) were fixed in a 40 mM-sodium cacodylate buffer at pH 7.4 containing 2% paraformaldehyde and 2.5% glutaraldehyde. They were postfixed in 2% osmium tetroxide, stained with saturated aqueous uranyl acetate and embedded in TAAB resin containing dodecenylsuccinic anhydride and methyl nadic anhydride as hardeners and DMP30 as accelerator. The silver-gold thickness sections obtained were examined in a Philips EM300 electron microscope.

Specimens for Alcian blue 8GX histochemistry were fixed in buffered formal saline with 0.5% cetylpyridinium chloride, embedded in paraffin wax, sectioned at 8 or 12 μm and then stained with 1% Alcian blue in pH 2.2 HCl in distilled water as previously described (Vanroelen *et al.* 1980b). The stained sections were mounted in DePeX and viewed under bright-field illumination using a Kodak No. 25 red filter.

RESULTS

1. *Electrophysiological studies*(a) *Potential measurements*

Blastoderms *in ovo* at stage 3–4 impaled with microelectrodes gave the following results (illustrated in Fig. 2):

(i) *Negative potentials.* The first event recorded upon penetration of any embryo with an electrode of 12 M Ω or more was a more or less stable negative potential, averaging -33 ± 8 mV (s.d.) ($n = 50$ impalements, 13 embryos). A finer tip (higher resistance) usually gave a more stable, higher amplitude result.

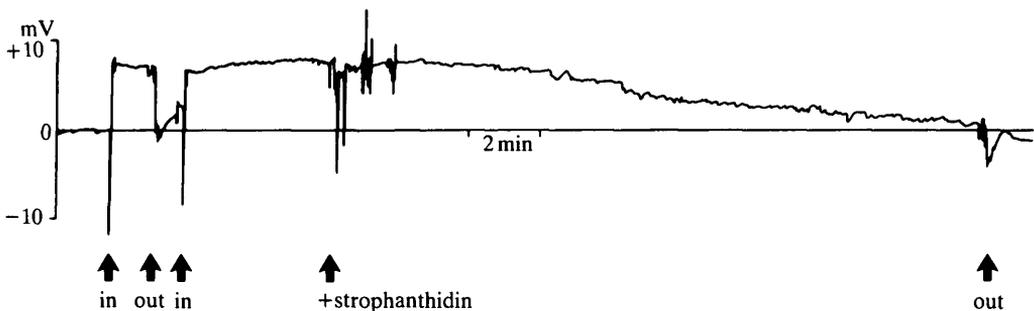


Fig. 2. Record from impalement *in ovo* of a stage-3⁺ embryo using microelectrodes and effect of strophanthidin. As the electrode is lowered, a brief negative transient is seen ('in'). With further lowering, a stable positive potential of about 9 mV is recorded. The phenomenon is repeatable at the same spot (second 'in'). After addition of strophanthidin at 10^{-5} M by injection into the yolk, the potential begins to decrease by about 5 min following the injection. The final 'out' demonstrates that the decrease in potential due to spontaneous drift is only about 1 mV. The spikes after the '+strophanthidin' mark are due to noise during injection.

Broken electrodes could not record stable negative potentials. The negative potentials are probably resting potentials recorded from within epiblast cells.

Several impalements of the yolk were made, beyond the edge of the blastoderm. In these an area of very high resistance ($\sim 10^8 \Omega$) and increased electrode noise was encountered.

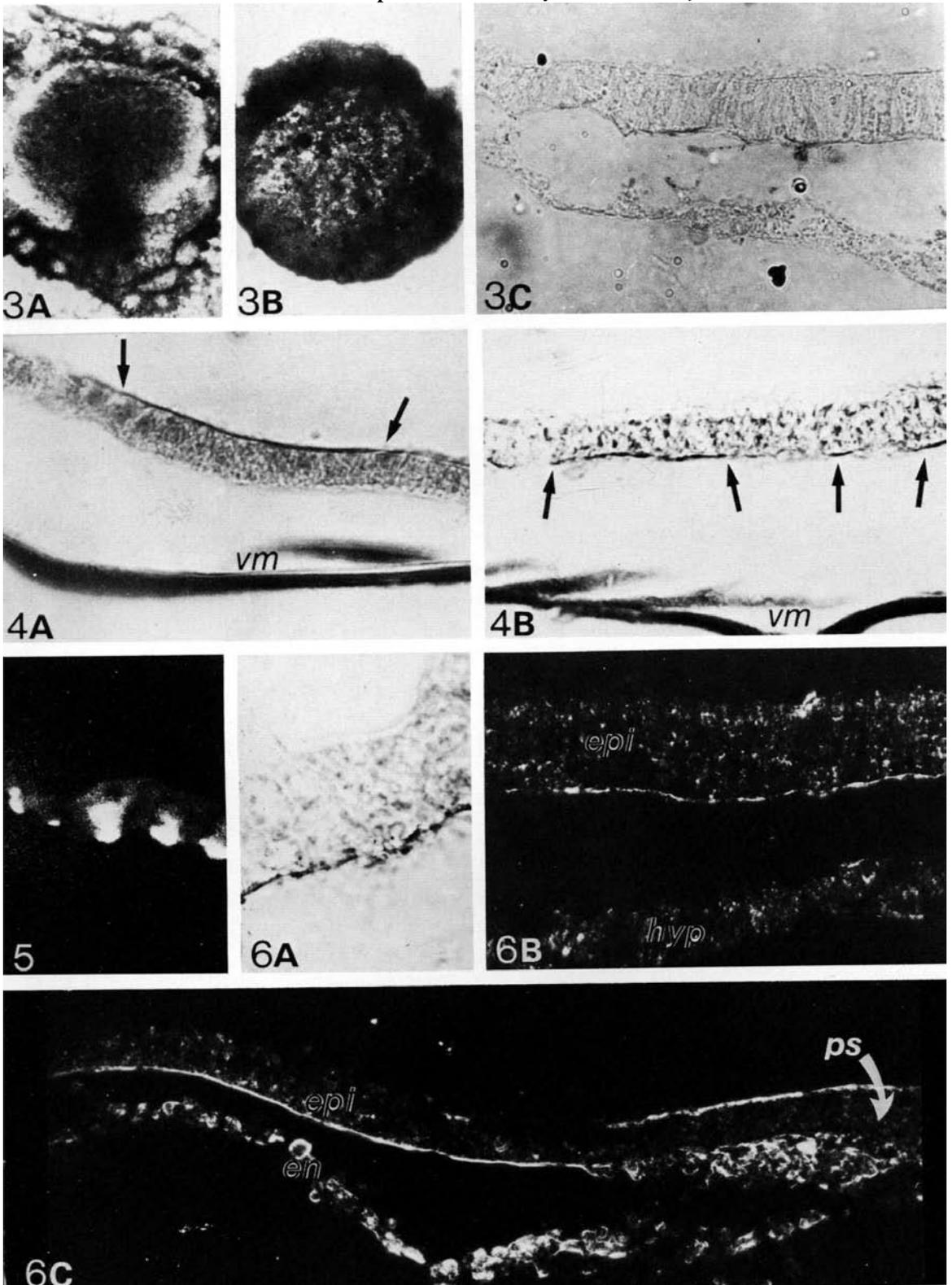
(ii) *Positive potentials.* With continued lowering of the electrode after recording a negative potential, or immediately upon entering an embryo with a broken electrode, a positive potential was observed, averaging $+16 \pm 5$ mV (s.d.) ($n = 74$ impalements, 31 embryos). Unlike the negative potentials described above, these positive potentials could be stably and repeatably recorded even with broken, very low-resistance electrodes. No consistent variations in this potential were observed between different regions of the blastoderms. Values tended to cluster within a few mV in each embryo. Variations are not likely to be due to impalement damage, as little difference was seen between results obtained with broken or unbroken electrode tips. In a few embryos, when the electrode was lowered well below where the first positive potential was measured, an area several mV more positive was encountered along with an abrupt increase in electrode noise, followed by a lower Ω region of still more noise. This was not observed when broken electrodes were used. The resistances

Fig. 3. Effect of strophanthidin on development. (A) Control embryo grown in New culture from stage XII in the presence of 10% ethanol. Note the normal development of a primitive streak. $\times 20$. (B) Embryo exposed to 5×10^{-6} M-strophanthidin at stage XII and grown in New culture for a similar length of time. No subsequent development of axial organs has taken place. $\times 20$. (C) Section through embryo in (B) stained with Alcian blue. Although the structure of the tissues seems unaffected, there is a marked reduction in Alcian blue-positive materials (c.f. Fig. 4A). $\times 240$.

Fig. 4. Effects of electrical stimulation on the position of Alcian blue-positive materials. (A) Control embryo grown in a 'mini-Ussing' chamber for 5 h. Note the position of the Alcian blue-positive materials (arrows) at the basal side of the tissue (top of picture), away from the vitelline membrane (vm). $\times 140$. (B) Epiblast stimulated for 3 h with 25 mV, apical side positive. All the Alcian blue positivity is now present at the apical side of the tissue (bottom of photograph, arrows), towards the vitelline membrane (vm). $\times 140$.

Fig. 5. Localization of the $\text{Na}^+\text{-K}^+\text{-ATPase}$ by indirect immunofluorescence. Although the fluorescence is patchy, it is localized at the basal side of the epiblast. Stage-XIV embryo, $\times 140$.

Fig. 6. Autoradiography after exposure to [^3H]Jouabain. (A) Section through stage-XIV embryo, phase-contrast optics, focused on silver grains in emulsion. The grains are localized only at the basal ends of the cells. Using these optics, intracellular yolk granules cannot be confused with silver grains in the emulsion (c.f. Fig. 6B). $\times 400$. (B) Same section as in Fig. 6A. Dark-field optics. The small bright areas inside epiblast (epi) and hypoblast cells represent reflections from small intracellular yolk granules. (c.f. Fig. 6A). $\times 330$. (C) By stage 3, the radioactivity is still basally located in lateral regions of the epiblast but appears at the apical side in the proximity of the primitive streak (ps). Mesoderm cells and endoderm cells show radioactivity all over their surface. hyp, hypoblast; en, endodermal layer. Dark-field illumination. Note that using these optics the silver grains in the emulsion appear white in the micrographs. $\times 150$.



Figs 3-6

measured (by current injection) while such increased noise was encountered ranged in tens of $M\Omega$. Neither the negative nor the positive potentials were obtained when impaling fixed embryos.

(b) *Effect of inhibitors*

When strophanthidin (final conc. = $10^{-5} M$) was injected into the yolk of stage 3 blastoderms *in ovo* ($n = 7$), the positive potential began to decrease within 5–8.5 min, reaching almost zero mV (a +1–2 mV residual voltage was usually present) within 30–40 min. A record from one such experiment is shown in Fig. 2. This effect was not mimicked by injection of the same quantity of solvent (10% ethanol in Pannett-Compton saline) into the yolk. Attempts to wash out the inhibitor were not successful due to difficulty in maintaining the impalements during this operation.

Strophanthidin at concentrations between 10^{-9} and $10^{-4} M$ was added to stage-XI to -3 blastoderms in New (1955) culture ($n = 47$). Concentrations higher than $5 \times 10^{-6} M$ inhibited the formation of the primitive streak in the earliest embryos (stage-XI to -XIII) (Fig. 3), and in a few cases (3/14) caused regression of the axis in the later ones. In histological sections, the structure of the individual tissues did not appear to be disrupted, but Alcian blue staining of the basal side of the epiblast was reduced or absent (c.f. Figs 3 & 4A, and see 'Normal Morphology' below). In most cases where the embryos were grown in the presence of the inhibitor it was possible to overcome its effects by washing the blastoderms thoroughly with saline and re-incubating overnight at $37^\circ C$ in the normal manner. Embryos cultured with strophanthidin at concentrations higher than $5 \times 10^{-4} M$ showed a disruption in the structure of the epiblast and hypoblast, likely to be due to a non-specific 'detergent' effect of the inhibitor. Attempts to culture embryos in sodium-free medium have not so far been successful, as the embryos tend to irreversibly lose their structural integrity.

(c) *Reversed polarity*

In this study, epiblasts at stage XIV or stage 2 were used. In the latter, the early primitive streak was excluded from the experimental region of the chambers.

(i) *Normal morphology.* Epiblasts cultured *in ovo*, by New (1955) culture or

Fig. 7. Epiblasts grown in mini-Ussing chambers with and without stimulation. (A) Semithin plastic section of control epiblast grown in chamber for 3 h. Note the predominantly basal position of the nuclei and the larger size of intercellular spaces towards the basal ends of the cells. $\times 650$. (B) Semithin plastic section of epiblast stimulated for 3 h with +25 mV. After stimulation, the nuclei lie predominantly towards the apical side of the cells and the intercellular spaces at the basal side are much reduced in size. $\times 650$. (C) Semithin plastic section of epiblast stimulated for 3 h with +25 mV. Two mitotic figures can clearly be seen (arrows). $\times 1100$. (D) Low-power electron micrograph of control epiblast. Note the large basal intercellular spaces. $\times 2800$. (E) Low-power electron micrograph of stimulated specimen. Note the close apposition between the cells. $\times 2800$.

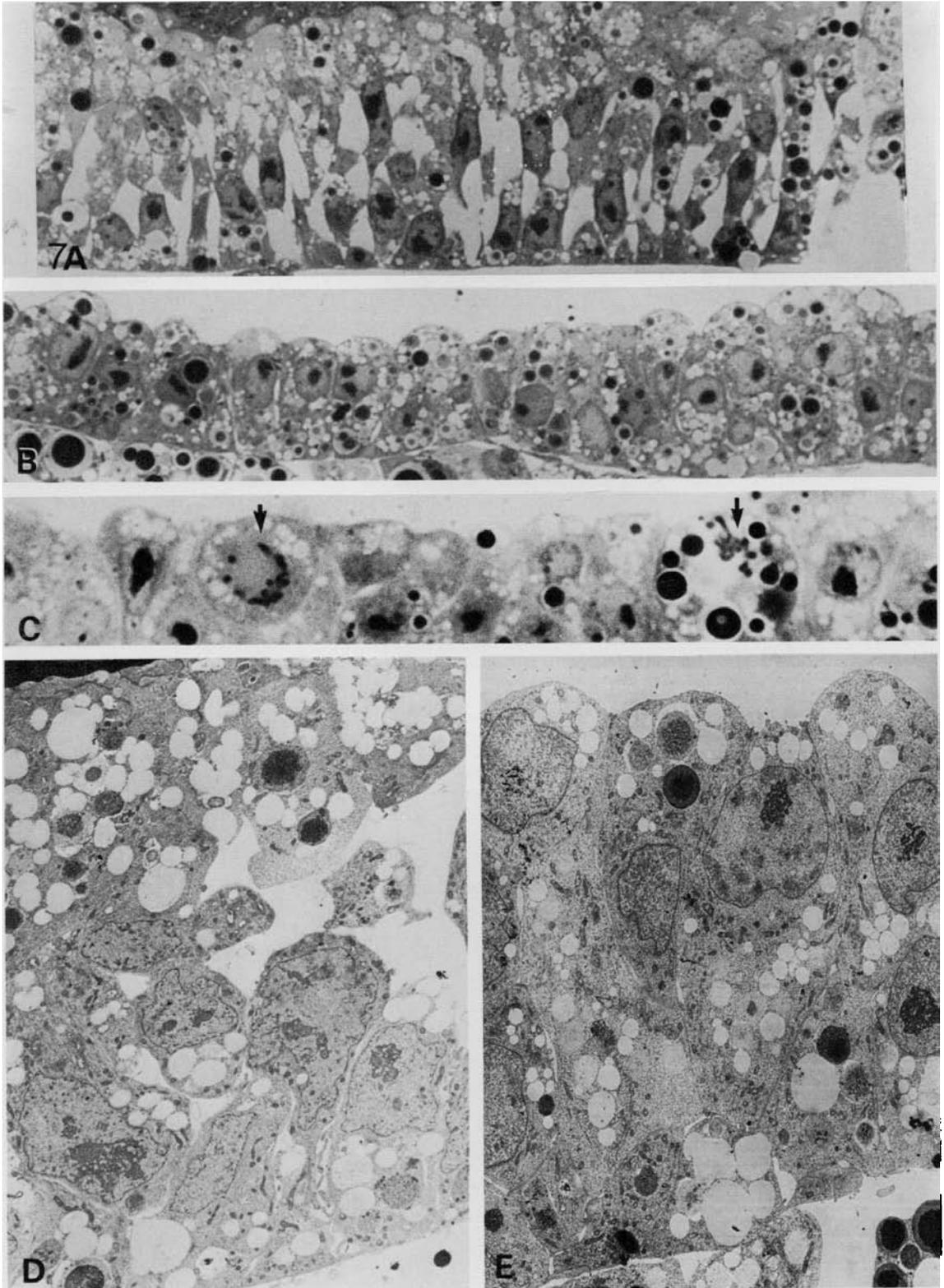


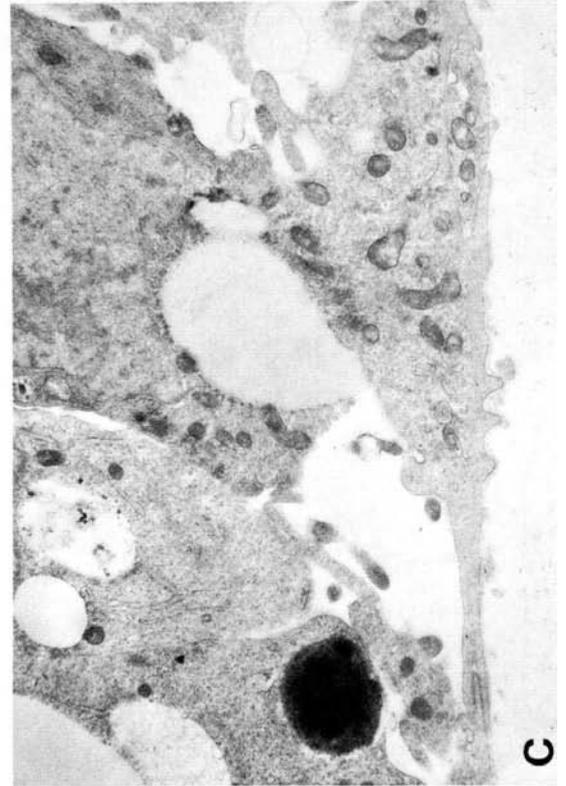
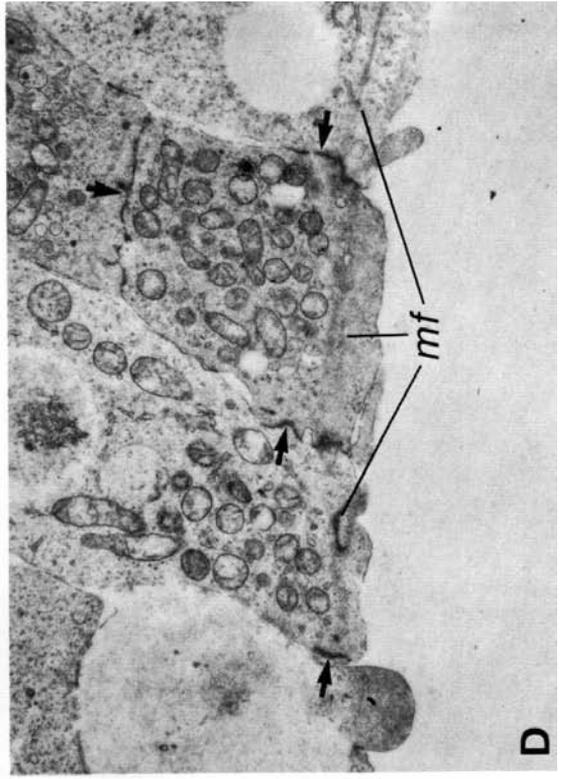
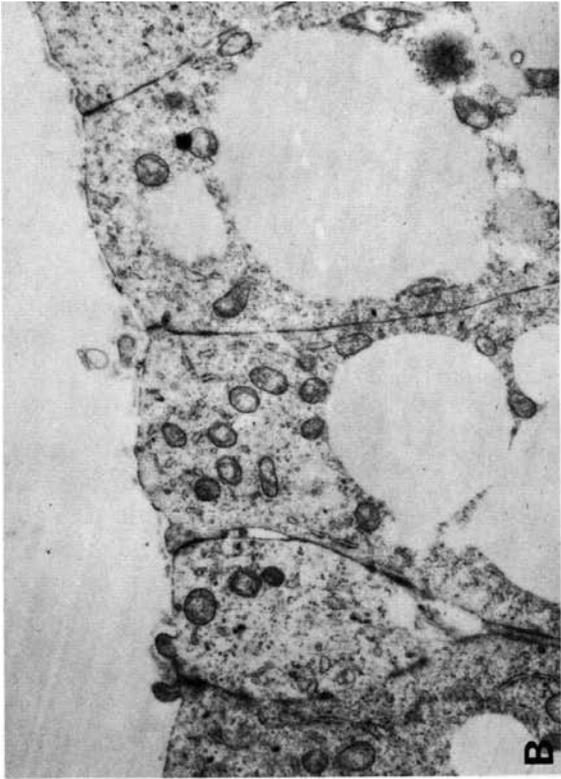
Fig. 7

in mini-Ussing chambers showed Alcian blue-positive materials at their basal side (Fig. 4A), correlated with loose electron-dense materials seen in the electron microscope (Fig. 8C). The apical ends of the cells were surrounded by electron-dense junctions and microvilli (Fig. 8A). Microfilament bundles are often associated with these junctions. The intercellular spaces were more prominent near the basal ends of the cells (Figs 7A & D) and the nuclei were predominantly located basally (Figs 7A, D).

(ii) *Reversed polarity.* Epiblasts cultured in mini-Ussing chambers and stimulated with their apical side 15–30 mV positive at the start of the experiment showed a shift in the location of Alcian blue-positive materials from the basal side of the tissue to the apical side after only some 60–90 min stimulation (Fig. 4B). This effect was seen in all 51 cases studied. The Alcian blue-positive material was sensitive to bovine testicular hyaluronidase, demonstrating that it contained glycosaminoglycans. The optimal pH for staining was 2.2. After 3 h stimulation the staining remained at the apical side of the cells even if the epiblasts were cultured for 12 h or more in the absence of stimulation. The electrical resistance across the sheets of tissue varied between $200 \Omega \text{ cm}^2$ and $9.7 \text{ K}\Omega \text{ cm}^2$ (mean = $2.0 \pm 1.1 \text{ K}\Omega$) before the start of the experiments, but it fell to about 1–10 % of its initial value by 30–45 min of stimulation; by 60–90 min it had returned close to the starting value (resistance measured in 12 experiments). This suggests that intercellular junctions are first broken and later reform.

Epiblasts stimulated for 3 h with the polarity of the voltage (15–20 mV at the start of the experiment) the same as in the embryo (basal side positive) did not show any signs of change in terms of the location or intensity of Alcian blue staining. Use of higher (20–30 mV) levels of initial voltage for over 3 h sometimes led to a reduction in the intensity of staining. The use of long (25 cm) agar bridges between the electrodes and the culture medium to reduce possible presence of electrode products did not affect the changes described. Epiblasts stimulated (apical side positive) at 4 °C showed no change in resistance, or in the position of Alcian blue positivity with respect to controls. However, if epiblasts were stimulated at 4 °C for 3 h and then placed at 37 °C for a further 3 h in the absence of stimulation ($n = 4$), areas of Alcian blue positivity were found at the apical surface of the tissue. These results indicate that although electrophoresis of extracellular materials is not alone responsible for the reversal, the establishment of a new polarity can be induced with little or no energy expenditure by the cells.

Fig. 8. Electron micrographs of control and epiblasts stimulated with +25 mV for 3 h. Control specimens were grown in the chambers for the same length of time. (A) Control epiblast, apical side. Note the apical tight junctions and the microvilli, most of which are sectioned transversely. $\times 14\,300$. (B) Stimulated epiblast, apical side. Note the close apposition of the cells and the extracellular matrix overlying the junctions. $\times 14\,300$. (C) Control epiblast, basal side. Note the large intercellular spaces and the extracellular matrix (c.f. Fig. 8B). $\times 14\,300$. (D) Stimulated epiblast, basal side. Note the presence of many junctions (arrows) and the microfilament bundles which insert into them (mf). $\times 14\,300$.



The morphological effects of stimulation were not restricted to the localization of Alcian blue-positive materials. In specimens stimulated for 3 h with 25 mV examined by TEM intercellular junctions could be found at both ends of the cells (c.f. Figs 8A and 8B, D, E). The electron density of these junctions in both controls and experimental specimens resemble the tight junctions previously described in the early chick embryo (Bancroft & Bellairs, 1974; Bellairs *et al.* 1975; Buck *et al.* 1976; Revel *et al.* 1973; Sanders, 1973). Furthermore, the newly formed basal junctions often displayed microfilament bundles inserting into them (Fig. 8D). The remaining apical junctions in stimulated specimens were often covered by basal lamina-like extracellular matrix (Figs 8B, E) which is probably the same material which stained with Alcian blue in the experiments described above. The normal basal extracellular matrix was not present near the basal ends of the stimulated cells (c.f. Figs 8B, D). Other features included a disappearance of the control cells' apical microvilli in stimulated specimens (c.f. Figs 8A and 8B) and a shift in the position of the nuclei from a more basal

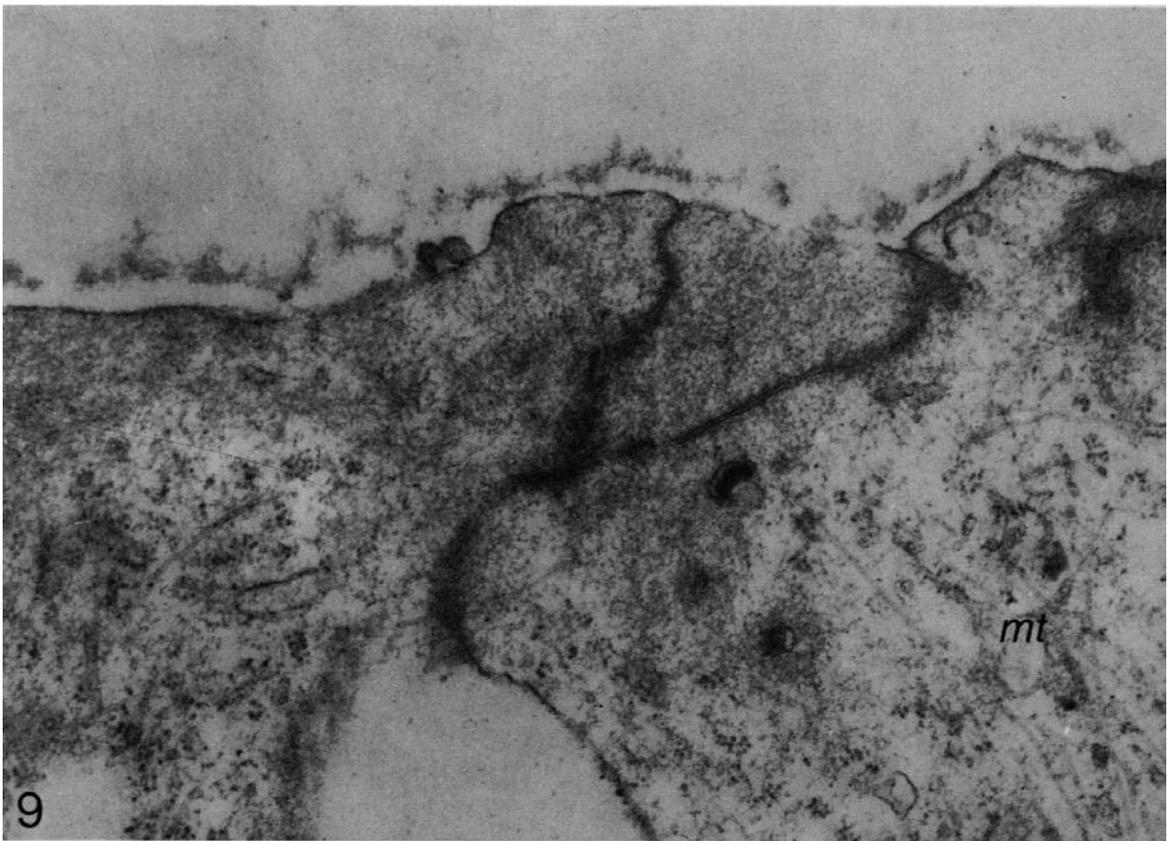


Fig. 9. Electron micrograph of an epiblast stimulated with +25 mV for 3 h. Apical side. The extracellular matrix can be clearly seen overlying the intercellular junctions which remain at this side of the tissue. Note the presence of microtubules (mt). $\times 54\,600$.

position in the controls to a more central or apical location in stimulated cells (Fig. 7).

An attempt to quantify this shift from photographs of semithin plastic sections by scoring nuclear position in the apical and basal halves of each cell revealed that in controls ($n = 4$. 168 cells), 15.5 % of the nuclei were apically located and 84.5 % basally, whereas in experimental embryos stimulated for 3 h with 25 mV ($n = 3$. 132 cells), 63.6 % were placed apically and 36.4 % basally. Mitotic figures were sometimes seen in both control and stimulated specimens (Fig. 7C).

2. Sodium transport

(a) Sodium fluxes

Stage-3 epiblasts were used for this study. The primitive streak was excluded from the measurement region of the chambers.

^{22}Na was administered to either the basal or the apical side of cultured sheets of epiblast in mini-Ussing chambers and samples removed at intervals from the opposite side of the tissue.

Figs 10–11 show the results obtained. It can be seen (Fig. 10) that transport is essentially uni-directional, in the apical→basal direction. This apical→basal

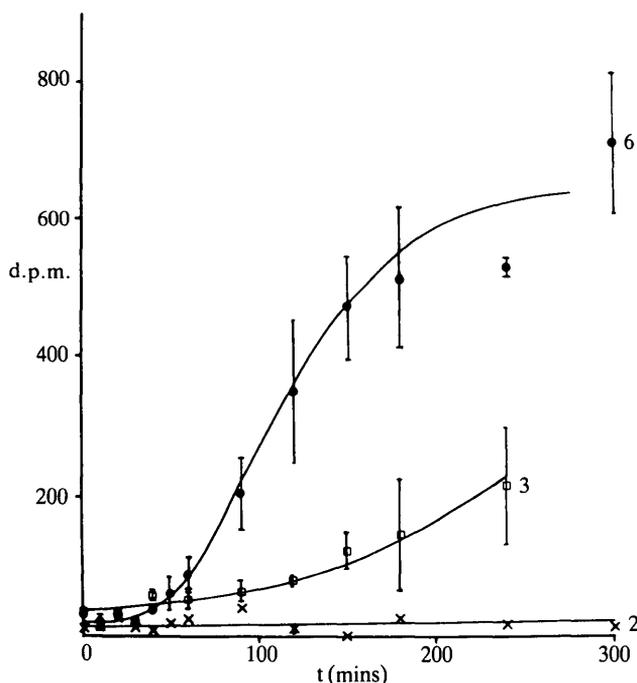


Fig. 10. ^{22}Na fluxes in epiblasts cultured in mini-Ussing chambers. Filled circles, apical→basal; crosses, basal→apical; squares, apical→basal in the presence of 10^{-5} M-strophanthidin. The bars represent the standard error of the mean. The numbers at the end of each line, the number of replicates for each of the experiments. The ordinate represents d.p.m. per mm^2 .

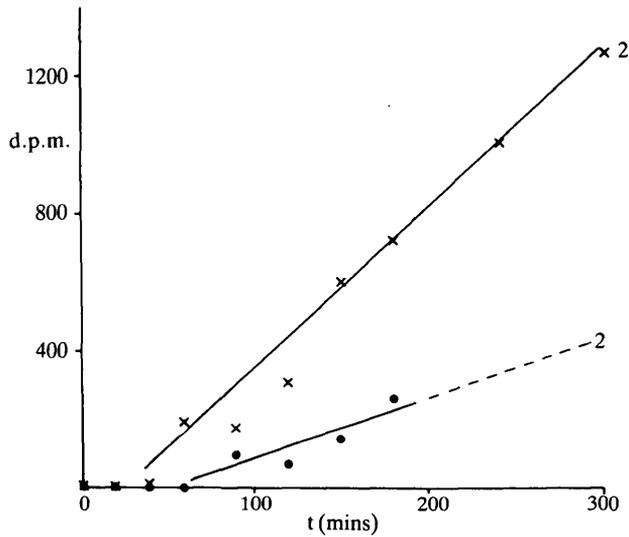


Fig. 11. ^{22}Na fluxes in experimentally reversed specimens. These had been stimulated for 3 h with an initial voltage of 24 mV, apical side positive. Filled circles, apical→basal; crosses, basal→apical. The predominant direction of transport is the reverse of that in controls. The numbers represent n for each experiment.

flux was considerably reduced by 10^{-5} M-strophanthidin, indicating that more than 50 % of the transport is due to activity of the sodium pump. Strophanthidin did not affect basal→apical flux.

Fig. 11 shows the results of the same experiments performed on epiblasts with reversed morphological polarity. In these experiments epiblasts were stimulated for 3 h prior to labelling, and then cultured in the absence of further stimulation. It can be seen from the figure that apical→basal flux is decreased and basal→apical flux is considerably increased, and the net direction of transport is completely reversed, although the rates are closer to each other than in control specimens, and the flux plot in the experimental specimens approaches a linear form rather than a sigmoid. Both the apical→basal and the basal→apical fluxes were strophanthidin sensitive, which shows that the increase in net flux is not due to increased diffusion alone.

From these experiments it was possible to establish a number of parameters relating the transport of sodium to the number of pumps from the [^3H]ouabain-binding experiments, shown in Table 2. For these calculations the slope of the transport curves between 60 and 180 min was used, assuming linearity within this range. From the table it can be seen that the net rate of sodium transport per cell or per cm^2 of tissue is about the same in unstimulated (apical→basal) or reversed (basal→apical) specimens, but the rate of transport per pump appears to be increased. It should be borne in mind, however, that this observation could represent an artefact generated by the relatively low number of ouabain-binding sites in the reversed specimens (Table 1).

Table 1. Results from [^3H]ouabain binding to epiblasts cultured in mini-Ussing chambers

		d.p.m. $\text{cm}^{-2} \times 10^5$	Difference	sites cm^{-2}	sites/cell	sites/ μg DNA	n	
Normal	Basal	0 K ⁺ 15 mM K ⁺	5.1 \pm 0.4 0.9 \pm 0.06*	4.2	5.7 $\times 10^{12}$	3.2 $\times 10^6$	1.8 $\times 10^{11}$	8
	Apical	0 K ⁺ 15 mM K ⁺	0.2 \pm 0.03 0.1 \pm 0.08	0.1	—	—	—	8
Reversed (37°C)	Basal	0 K ⁺ 15 mM K ⁺	0.2 \pm 0.02 N.S.B.	0.2	3.1 $\times 10^{11}$	1.8 $\times 10^5$	9.8 $\times 10^9$	5
	Apical	0 K ⁺ 15 mM K ⁺	0.4 \pm 0.05 N.S.B.*	0.4	5.4 $\times 10^{11}$	3.0 $\times 10^5$	1.7 $\times 10^{10}$	6
Clamped (4°C)	Basal	0 K ⁺ 15 mM K ⁺	5.7 \pm 0.05 N.S.B.*	5.7	7.5 $\times 10^{12}$	4.2 $\times 10^6$	2.4 $\times 10^{11}$	6
	Apical	0 K ⁺ 15 mM K ⁺	0.1 N.S.B.	0.1	—	—	—	3

The values under d.p.m. represent means \pm standard error. The others represent mean number of net ("specific") sites. Whilst control epiblasts have virtually all their "specific" binding sites at their basal ends, experimentally reversed epiblasts show a considerable increase in the number of apical sites and a decrease in the number of basal ones. Specimens stimulated at 4°C are not significantly different from controls. N.S.B., not significantly different from background. *, $P < 0.05$.

Table 2. ^{22}Na transport rates. The table shows the rate of pumping calculated from ^{22}Na flux measurements and the number of pumps present from [^3H]-ouabain-binding experiments (Table 1)

	Normal (net)	Reversed (net)
^{22}Na atoms/cell/h	2.3×10^{13}	2.7×10^{13}
^{22}Na atoms/pump/h	6.8×10^6	8.9×10^7
^{22}Na atoms/cm ² /h	4.0×10^{19}	4.8×10^{19}

The values represent the net rates of basal \rightarrow apical flux after subtracting the "back flow" in the apical \rightarrow basal direction. In reversed polarity specimens, the pumps appear to transport the ion more actively than in the controls.

(b) *Intraembryonic sodium and volume measurements*

From the amount of ^{22}Na remaining within the tissue after performing the transport studies the following parameters could be estimated:

(i) In control specimens (stage 3) the total apically or basally filled intratissue (comprising intracellular plus intercellular) compartment contained 7.1 nmoles/epiblast.

(ii) The intercellular space in stage 3 *area pellucida* epiblast (determined with [^{14}C]mannitol) averaged $0.1 \mu\text{l}$ ($n = 3$). The intercellular volume in the *area opaca* (including epiblast and germ wall) was between 0.7 and $1.5 \mu\text{l}$. From these estimates, the mean intercellular sodium concentration in the stage 3 *area pellucida* epiblast could be calculated as 71 mM. The intracellular concentration cannot be determined from these experiments, as the intracellular volume is not known. Attempts to resolve this using various ion-sensitive microelectrodes have so far been unsuccessful.

(iii) In *area pellucida* epiblasts (stage 3) with reversed morphological polarity the volume of intercellular spaces measured $0.08 \mu\text{l}$ ($n = 2$). The apically labelled intratissue (i.e. intercellular plus intracellular) sodium content in these specimens was 6.6 nmoles/epiblast, whilst the basally labelled pool was 131 nmoles/epiblast. As this difference does not appear to be due to an increase in the volume of intercellular space in the reversed specimens, it may therefore be indicative of an increase in the number of sodium pumps present (see below), as this would lead to the cells filling up with sodium faster than control specimens.

Embryos at stage 3 were incubated in New (1955) culture in the presence of ^{22}Na . After this (see Materials & Methods) some were washed whole and some were first dissected to wash out the contents of the blastodermic cavity. Comparison of these two measurements revealed the following parameters:

(i) The amount of sodium present in the blastodermic cavity was determined as 56 nmoles. Using [^{14}C]mannitol it was established that the total volume of the blastodermic cavity (i.e. that between the epiblast and the hypoblast) at stage 3

averaged $0.6 \mu\text{l}$ ($n = 6$). This gives a crude estimate of the concentration in the cavity of 94 mM for stage 3 embryos.

(iii) From a time-course experiment ($n = 8$, stage 3) it was established that the cavity filled up with ^{22}Na very rapidly, reaching a steady state in less than 60 min when the label was administered apically.

3. Localization of the sodium pump

(a) Tritiated ouabain autoradiography

Fig. 6 shows the localization by autoradiography of [^3H]ouabain binding to early chick embryos grown in New (1955) culture. A total of 11 embryos were studied, at either stage XIV, stage 3 or stage 4. Figs 6A, B show the typical pattern obtained in embryos prior to the appearance of the primitive streak. The radioactivity in the epiblast is restricted to the basal regions of the cells. This situation holds in all regions of the embryos observed at this stage (stage XIV). The bright areas inside both epiblast and hypoblast cells in the dark-field micrographs correspond to small yolk granules which appear light in dark-field optics. Fig. 6A is a bright-field micrograph of the same section as in Fig. 6B showing the basal location of the grains in optical conditions where the intracellular yolk cannot be confused with labelling. At later stages (stage 3–4) the epiblast still displays ouabain binding in basal regions away from the primitive streak. In the immediate vicinity of the streak, however, most of the radioactivity is localized over the apical borders of the epiblast cells (Fig. 6C). The endoderm cells and mesoderm cells at the primitive streak and lateral regions show grains all over their surface and no asymmetry of binding is apparent (Fig. 6C).

(b) Immunological localization

Localization of the Na-K-ATPase using antibody gave less clear results than [^3H]ouabain autoradiography, probably due to limited cross reactivity between the chick pumps and the antibody which had been raised against a rabbit-derived preparation. Nevertheless in a few cases (6/47) clear but 'patchy' fluorescence was observed associated with the basal side of epiblast cells at stage XIV (Fig. 5). The remaining embryos showed much less fluorescence overall, and none of it could be localized to any specific part of the cells. This diffuse binding probably represents cross reactivity with other intracellular ATPases. None of the controls (see Methods) showed any localized fluorescence.

(c) Quantitative determination of number of pumps

A total of 16 normal epiblasts and 11 with experimentally reversed morphological polarity were studied. All were at stages 2–3, and their primitive streaks were excluded from the measurement area of the chambers. [^3H]ouabain was administered to either the apical or the basal side of epiblasts cultured in mini-Ussing chambers in either 15 mM or 0 mM K^+ medium. The rationale for this is

that the difference between the counts bound under the two conditions gives an estimate of specific ouabain binding to the pump, as binding is competitively inhibited by potassium (Simmons, 1981). Table 1 summarizes the results obtained. From this it can be seen that virtually all 'specific' binding is localized at the basal ends of the cells.

The situation in specimens with reversed dorsoventral morphological polarity (stimulated with 25 mV for 3 h, apical side positive) is not as straightforward. Here there appears to be less difference in binding to each side of the tissue, and there is some reduction in the total number of binding sites (total apical *plus* total basal) with respect to controls. Nevertheless, there is a slight but significant increase in the number of apical 'specific' binding sites in the stimulated specimens. These results indicate that in the specimens with reversed morphological polarity some of the pumps may become located at sites within the intercellular space which are not accessible to the tritiated ouabain. Alternatively, some of the pumps may be removed from the basal membrane of the cells at a faster rate than new pumps are synthesized at the opposite ends of the cells. On the other hand, longer stimulation (up to 6 h), or culture of the epiblasts for longer periods of time (up to 18 h) after stimulation never led to a greater total number of binding sites, or to the difference between apical and basal binding becoming more marked. Epiblasts stimulated at 4°C showed no change with respect to controls.

DISCUSSION

The foregoing results indicate that: (a) the epiblast of early chick embryos possesses a sodium pump which is located at the basal side of this tissue; (b) a measurable positive potential of some 15 mV (basal side positive) is present across the epiblast; (c) both the potential and sodium transport are sensitive to strophanthidin; (d) electrical stimulation with apical side positive (the reverse polarity to the voltages measured across the epiblast in the embryo) leads to a rapid reversal in the position of various morphological polarity markers and (e) the reversal of morphological polarity is accompanied by at least a partial reversal of physiological functions.

1. *Electrophysiology*

The results of our electrophysiological study clearly confirm that the epiblast maintains a voltage across itself with its ventral (basal) side positive.

The mechanical delicacy of the negative potentials, extending to outright unobtainability with very low-resistance, large-tip electrodes, the difficulty in imagining any other such negative potential source in the tissue and the similarity of the values to those of Sheridan (1966, 1968) all support the conclusion that it is intra-epiblastic and represents a resting intracellular potential.

The width of the space where the positive potential was encountered, as

suggested by distance measurements (MacKenzie, 1980) and its accessibility with broken electrode tips, unlike the negative area, suggests an extracellular, transepithelial location. Thus the chick epiblast maintains a potential, basal side positive, of some 16 mV across itself. This is notably similar to the size and polarity of that maintained by mouse trophoctoderm (+21 mV) (Benos, 1981, and references therein). The frog skin maintains an even larger positive potential across itself (Ziegler, 1977 and references therein).

The lack of successive negative transients indicative of the crossing of more than one germ layer is probably due to the fact that the thin, flat definitive endoblast cells are beyond the mechanical delicacy of the system.

The area of increased electrode noise which was encountered below the positive zone probably represented the impalement of one or more yolk spheres, as similar noise was seen during yolk impalement. This is further supported by the fact that increased noise was not seen with broken tip electrodes. On the contrary, with increased penetration of the yolk with broken electrodes, a slow, graded fall off of the positive voltage was observed, as if moving an electrode away from a point voltage source through a volume conductor.

2. *Inhibitor effects*

The observation that strophanthidin can inhibit the formation of the primitive streak and other axial structures in the early embryo, and considerably reduce sodium fluxes and the positive potential is indicative that activity of the sodium pump may be of importance in the control of the developmental processes leading to the formation of these structures. As with all inhibitor studies, however, caution should be exercised in the interpretation of these results, as there is no absolute guarantee that the effect on development is being achieved directly through inhibition of sodium pump activity. Even if the effect was due to pump inhibition alone, this might be leading to such a general disruption of cell metabolism that developmental processes could be affected through this.

3. *Localization of the pump*

Our results show that the sodium pump (as determined by [³H]ouabain binding, autoradiography and indirect immunofluorescence) is located at the basal side of the epiblast at preprimitive streak stages. At primitive streak stages, however, the pumps are still located at the basal side of the epiblast in lateral regions but at the primitive streak they appear at the apical side of the tissue. This observation suggests that whilst sodium is pumped into the interior of the blastoderm in lateral regions, the primitive streak cells pump the cation out of the blastodermic cavity. This is entirely consistent with the pattern of extracellular currents which has been measured using the vibrating probe (Jaffe & Stern, 1979). Unlike the original suggestion of a passive leak out of the streak, however, there appears to be active transport of the cation out of the blastodermic cavity.

There is an interesting parallel between this pattern and a similar reversal

described by Vanroelen *et al.* (1980*a-c*; see particularly fig. 2a in 1980*a* and fig. 2b in 1980*c*). They established that newly synthesized extracellular materials are incorporated basally in the lateral epiblast but apically at the primitive streak, as determined by $^{35}\text{SO}_4$ and $[^3\text{H}]$ glucosamine incorporation and autoradiography. Fibronectin and laminin localization also appear to follow this pattern, as has recently been shown by indirect immunofluorescence (Mitrani & Farberov, 1982) that they are present in lateral regions of the epiblast but not at the primitive streak at about stage 2-3. These results point towards a possible connection between the localization of basal lamina components (Sanders, 1979; Vanroelen *et al.* 1980*a-c*; Wakely & England, 1979) and the position and function of the sodium pumps in the chick epiblast.

4. Sodium transport

The number of 'specific' ouabain-binding sites per cell in the present study correlate very well with those reviewed by Benos (1981) for other transporting epithelia. The amount of sodium transported per pump per unit time, however, appears to be higher in the chick blastoderm (this study) than in the rabbit (Benos, 1981), by a factor of 2.5. Together with the observation that the chick epiblast contains 10 times more pumps per cell than the rabbit, these observations indicate that the degree of specialization of the chick blastoderm for sodium transport is even greater than that of its rabbit counterpart.

The estimates of intercellular and blastodermic cavity sodium concentrations by the methods used in this study are by nature rather crude. One reason for this is that even small errors become magnified in calculations using the two different measurements (^{22}Na content and volume). Another important reason is that several assumptions have to be made for the calculation, e.g. that the proportion of labelled to unlabelled sodium inside the spaces to be measured is the same at equilibrium than that in the bathing medium. This assumption would lead to an underestimation of the sodium concentration in these spaces, as not all the sodium in these is likely to be exchangeable. Attempts to measure these parameters using ion-selective microelectrodes have not yet been successful.

5. Reversed polarity specimens

The present study shows that electrical stimulation (of similar magnitude but reverse polarity to the measured potentials) across chick epiblasts in culture can reverse their morphological polarity within a short time.

Jaffe (1977) was the first to suggest that extracellular voltage gradients within the normal physiological range might be capable of electrophoresing charged molecules within the plane of the plasma membrane of cells. Since then, this has been confirmed for a variety of systems (Poo, 1979*a,b*; see also Jaffe, 1979). In the present study, the measured positive voltage is at least one order of magnitude larger than the minimal voltage required for electrophoresis of most membrane-contained molecules as calculated originally by Jaffe. Although

neither the extracellular materials nor the ouabain-binding sites are completely displaced to the apical side by stimulation at 4 °C, as would be expected if either of these were being electrophoresed, there remains the possibility that these molecules are not capable of being moved through areas containing intercellular junctions. It has recently been shown (Martinez-Palomo, Meza, Beaty & Cerejido, 1980) that these cannot be induced to open at low temperature; this is also consistent with our resistance measurements of specimens stimulated at 4 °C, which did not decrease during stimulation.

Thus it is possible that stimulation might initially electrophorese the sodium pumps themselves to the apical part of the membrane of the epiblast cells. This interpretation would account for the behaviour of epiblasts which have been stimulated at 4 °C and then incubated at 37 °C in the absence of further stimulation. Electrophoresis of the pumps under these conditions, which does not require the expenditure of metabolic energy, would lead to a situation where the tissue provides its own 'voltage clamp' when restored to the incubation temperature. An alternative explanation is that another membrane component is being electrophoresed, such as a cytoskeletal element, which might determine the insertion of pumps and the production of basal lamina constituents on incubation at the higher temperature. These possibilities will be investigated in a future study.

It has been shown (Mauchamp *et al.* 1979; Nitsch & Wollman, 1980) that thyroid gland follicles in culture can also be induced to reverse their morphological apical-basal polarity by altering the serum concentration in the culture medium. Jaffe (1981) has recently pointed to a possible connection between this serum-induced reversal and Na⁺ fluxes consistent with the results in the present study. Furthermore, the polarity of the gut epithelium in the sea urchin embryo can also be reversed by changing the ionic composition of the bathing medium (Amemiya, Akasaka & Terayama, 1979). Thus, the role of ion transport in the control of morphological polarity in transporting epithelia may be a crucial and general phenomenon.

Our results point to a possible connection between the role of the primitive streak as a leak of sodium ions and extracellular electrical current on one hand, and the distribution of cellular markers of morphological polarity such as tight junctions and extracellular materials on the other. It is impossible to tell from the present data, however, just how the reversal of morphological dorsoventral polarity might be induced by the applied voltage, beyond the observation that the expression of the reversal requires an active participation by the cells. The observation that more protracted stimulation, under the present experimental conditions, cannot improve the degree of reversal of the physiological functions examined, indicates that other, as yet unknown factors, may be involved in the control of the spatial properties of these functions.

6. *Relevance to embryonic development*

Our results indicate that the positive voltage may be important for the control

of the positioning of morphological cell polarity markers and extracellular materials in different regions of the early chick epiblast. Another function of sodium transport at these early stages may be to facilitate the diffusion of water from the albumen into the embryonic region (see New, 1956; Ziegler, 1977) following the osmotic gradient generated by the sodium pumps. The water-transporting properties and their relation to ion fluxes in these embryos are currently under investigation.

Chick embryos are not unique among early vertebrate embryos in having well-developed sodium transport functions. Cross & Brinster (1970) and Cross (1971, 1973) have demonstrated, respectively, a transepithelial voltage and unidirectional sodium and chloride fluxes across the mammalian blastocyst. Amphibian embryos have been reported to rely heavily on sodium pump activity for neural differentiation (Messenger & Warner, 1979; Warner, 1973). Barth & Barth (1969) have reported that sodium entry is important for neural induction. In this animal, which has adapted to develop in a milieu of very low ionic strength, the sodium pumped by the cells appears to be derived from intra-embryonic stores (Slack & Warner, 1973). It thus appears as if sodium transport is an important physiological function which is used at very early stages of development in these three groups of vertebrates.

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