

**Birkbeck ePrints: an open access repository of the
research output of Birkbeck College**

<http://eprints.bbk.ac.uk>

Latter, G. V. and Baggott, G. K. (2002) Role of carbon dioxide and ion transport in the formation of sub-embryonic fluid by the blastoderm of the Japanese quail. *British Poultry Science* 43 (1) pp 104-116

This is an author-produced version of an article published in *British Poultry Science*. This version has been peer-reviewed but does not include the final publisher proof corrections, journal layout or journal pagination.

All articles available through Birkbeck ePrints are protected by intellectual property law, including copyright law. Any use made of the contents should comply with the relevant law.

Citation for this version:

Latter, G. V. and Baggott, G. K. (2002) Role of carbon dioxide and ion transport in the formation of sub-embryonic fluid by the blastoderm of the Japanese quail. London: Birkbeck ePrints. Available at:
<http://eprints.bbk.ac.uk/archive/00000224>

Citation for the publisher's version:

Latter, G. V. and Baggott, G. K. (2002) Role of carbon dioxide and ion transport in the formation of sub-embryonic fluid by the blastoderm of the Japanese quail. *British Poultry Science* 43 (1) pp 104-116

<http://eprints.bbk.ac.uk>

Contact Birkbeck ePrints at lib-eprints@bbk.ac.uk

The role of carbon dioxide and ion transport in the formation of sub-embryonic fluid by the blastoderm of the Japanese quail.

G.V. Latter and G.K. Baggott¹

Department of Biology, School of Biological and Chemical Sciences, Birkbeck College,
University of London, Malet St., London WC1E 7HX, UK.

Short title: CO₂, ions and sub-embryonic fluid

¹ To whom correspondence should be addressed.

ABSTRACT

2
4
6
8
10
12
14
16
18
20
22
24
26
28
30

1. The explanted blastoderm of the Japanese quail was used to explore the role of ions and carbon dioxide in determining the rate of sub-embryonic fluid (SEF) production between 54 and 72 hours of incubation.
2. Amiloride, an inhibitor of Na^+/H^+ exchange, at concentrations of 10^{-3} to 10^{-6} M substantially decreased the rate of SEF production when added to the albumen culture medium. N-ethylmaleimide, an inhibitor of V type H^+ ATPase, also decreased this rate but only to a small extent at the highest dose applied, 10^{-3} M. Both inhibitors had no effect on SEF production when added to the SEF.
3. The inhibitors of cellular bicarbonate and chloride exchange, 4-acetamido-4'-isothiocyanato-2,2'-disulphonic acid (SITS) and 4,4' diisothiocyanostilbene-2, 2'-disulphonic acid (DIDS), had no effect upon SEF production.
4. Ouabain, an inhibitor of Na^+/K^+ ATPase, decreased SEF production substantially at all concentrations added to the SEF (10^{-3} to 10^{-6} M). Three sulphonamide inhibitors of carbonic anhydrase, acetazolamide, ethoxzolamide and benzolamide, decreased SEF production when added to the SEF at concentrations of 10^{-3} to 10^{-6} M. Benzolamide was by far the most potent. Neither ouabain nor the sulphonamides altered SEF production when added to the albumen culture medium.
5. Using a cobalt precipitation method, carbonic anhydrase activity was localised to the endodermal cells of the *area vasculosa*. The carbonic anhydrase activity was primarily associated with the lateral plasma membranes, which together with the potent inhibitory effect of benzolamide, suggests the carbonic anhydrase of these cells is the membrane-associated form, CA IV.
6. The changes in SEF composition produced by inhibitors were consistent with the production of SEF by local osmotic gradients.
7. It is concluded that a Na^+/K^+ ATPase is located on the basolateral membranes of the endodermal cells of the *area vasculosa*, and that a sodium ion/hydrogen ion exchanger is located on their apical surfaces. Protons for this exchanger would be provided by the

hydration of CO₂ catalysed by the membrane-associated carbonic anhydrase. Furthermore,
it is proposed that the prime function of the endodermal cells of the *area vasculosa* is the
production of SEF.

INTRODUCTION

During the first few days of incubation a fluid accumulates beneath the avian embryo, the sub-embryonic fluid (SEF), that is produced by movement of water from albumen into the yolk sac (Romanoff, 1960, 1967; Simkiss, 1980, Ar, 1991). In the domestic fowl *in ovo*, a fluid is measurable beneath the blastoderm by 16-20h of incubation (Stern and Mackenzie, 1983; Stern, 1991). However, the maximum volume of SEF is found later: at 6 days of incubation in the domestic fowl (Deeming, 1989) and at 4.5 days in the Japanese quail (Babiker and Baggott, 1992); this fluid is also produced by the avian blastoderm when cultured on albumen (New 1956; Babiker and Baggott, 1995).

As *in ovo* SEF was found to have a sodium concentration greater than that of albumen (Howard, 1957; Babiker and Baggott, 1991, 1992) and it has been proposed that it is generated by a difference in osmolality between SEF and albumen (Howard, 1957; Ar, 1991). If this were the case, then a decrease in fluid production would be accompanied by a reduction in the difference in osmolality between SEF and albumen. However, when fluid production was inhibited experimentally in the quail blastoderm cultured on albumen, the volume of SEF was decreased but its osmolality and ionic composition did not change (Babiker and Baggott, 1995), an observation consistent with fluid production by local osmotic gradients (Diamond, 1979).

Indeed, if local osmotic gradients are responsible for production of SEF then the fluid secreted will be either isosmotic or hyperosmotic to albumen (Diamond and Bossert, 1967): the latter has been observed both *in ovo* (Howard, 1957; Babiker and Baggott, 1992) and in culture (Babiker and Baggott, 1995).

In the explanted blastoderm of the Japanese quail, the volume of SEF produced was dependent upon the sodium concentration of the albumen culture medium (Babiker and Baggott, 1995), and was decreased by amiloride, an inhibitor of sodium/hydrogen ion exchange. Although the volume produced was unaffected by inhibitors of anion transport across the plasma membrane, fluid production was decreased substantially by acetazolamide, an inhibitor of the enzyme carbonic anhydrase (Babiker and Baggott, 1995). Different ion- and fluid-transporting epithelia have been reported to utilise this enzyme to provide hydrogen ions for exchange with sodium ions across the plasma membrane, the protons being derived from hydration of carbon dioxide catalysed by the enzyme (Maren, 1967, 1988). In fluid-transporting cells of epithelia, carbonic anhydrase may be localised intracellularly (e.g. the turtle bladder; Fritsche *et al.*, 1991), plasma membrane-associated (e.g. the human gall-bladder; Parkkila *et al.*, 1996), or localised extracellularly (e.g. the kidney proximal tubule; Dodgson, 1991b). Also, whilst the endodermal cells of the *area vasculosa*, for example, possess characteristics of a fluid transporting epithelium, notably the long intercellular spaces with apical tight junctions (Mobbs and MacMillan, 1979), in the avian blastoderm the cell(s) responsible for SEF production have not been identified, although the factors influencing fluid production have been partially characterised (Babiker and Baggott, 1995). In our study, the objective was to define more exactly which ion-transporting mechanisms were essential for SEF production by explanted blastoderms in culture and to clarify the role of carbonic anhydrase, both by the use of sulphonamide inhibitors and by the localisation of enzyme activity histochemically, using the cobalt precipitation method of Hansson (1968). In addition, the histochemical localisation of carbonic anhydrase activity should permit the identification of those cells participating in SEF production by the avian blastoderm.

24

MATERIALS AND METHODS

26

Culture method

28 The method of blastoderm culture was based upon that of New (1955) as modified by Babiker and Baggott (1995) for the Japanese quail (*Coturnix coturnix japonica*). Fertile eggs were

incubated at 37.6 ± 0.1 °C for 24h before blastoderm explantation, which was performed in
2 Pannett and Compton (1924) salt solution under sterile conditions at 22°C in a laminar flow
cabinet (MDH, Andover, Hants). A blastoderm was dissected out attached to its vitelline
4 membrane, inverted, and a glass ring (14.3 mm internal diameter) placed centrally over the
blastoderm on the inner surface of the vitelline membrane. The explant, together with the glass
6 ring, was placed onto a circular glass supporter (of same internal diameter as glass ring)
located in a watch-glass. The watch-glass containing the explant was transferred to the base of
8 a Petri dish and the space beneath the glass supporter and blastoderm filled with thin albumen
(culture medium). A thin layer of sterilised double distilled water was added to the Petri dish to
10 maintain an atmosphere saturated with water vapour. The dish was then covered with a lid and
the culture incubated in an incubator at 37.6 ± 0.1 °C and c. 60 % r.h.

12 ***Measurement of SEF volume***

At both 54 and 72h of incubation the volume of SEF was estimated by the dye dilution method
14 of Babiker and Baggott (1995). At 54h of incubation, 20 µl of sterile amaranth dye solution
(400mg amaranth/100 ml of distilled water) was added to the SEF of an explant. After 30
16 minutes, 10µl of SEF was removed under sterile conditions and the culture returned to the
incubator. At 72 h of incubation the culture was again removed and a further 10 µl of SEF
18 removed. The protein in SEF samples was precipitated with trichloroacetic acid at a final
concentration of 10 % w/v, and the sample diluted before measuring the absorbance at 520 nm
20 using a Pye-Unicam PU6800 spectrophotometer (Babiker & Baggott, 1995). The apparent
hourly rates of fluid production (J_{ap} , µl/h) were calculated from the difference in volumes at 72h
22 and 54h of incubation divided by 18.

Measurement of sodium concentrations and osmolality

24 Sodium concentration was measured at both 54 h and 72 h of incubation with a Jenway PFP7
flame photometer calibrated with standard solutions of 50, 100 and 150 mM L⁻¹ sodium.
26 Samples, 10 µl of SEF or 10 µl culture medium, were diluted 1:250 with double distilled water

prior to measurement. Osmolality of samples, 5 μl of SEF or 5 μl culture medium, was estimated at both 54h and 72h of incubation using a vapour pressure osmometer (Wescor 5100B) calibrated with standards of 290 and 1000 mOsm Kg^{-1} .

4 ***Inhibitors of ion and fluid transport***

A synthetic salt solution (SSS) formulated from measurements of ionic concentrations of quail albumen was used as the carrier for the inhibitors (Babiker and Baggott, 1995). Inhibitors were added under sterile conditions to culture medium or SEF in separate experiments. For every inhibitor only the highest concentration, 10^{-3} M, was applied to the fluid (SEF or culture medium) where there was no discernible effect on fluid production. Amiloride or N-ethylmaleimide (NEM) was dissolved in SSS (2×10^{-2} M, or appropriately diluted) and 50 μl was added at 54h of incubation to 1 ml of culture medium to produce concentrations of 10^{-3} to 10^{-6} M amiloride. 10 μl of amiloride or NEM at 10^{-2} M in SSS was added to SEF at 54h of incubation to obtain a nominal concentration of 10^{-3} M, assuming a SEF volume of 100 μl . 4,4'-diisothiocyanostilbene-2,2'-disulphonic acid (DIDS), 4-acetamido-4'-isothiocyano-2,2'-disulphonic acid (SITS), or ouabain was dissolved in SSS (10^{-2} M, or appropriately diluted) and 10 μl was added to SEF at 54h of incubation to produce nominal concentrations 10^{-3} to 10^{-6} M, assuming a SEF volume of 100 μl . For each of these inhibitors dissolved in SSS at 2×10^{-2} M, 50 μl was added to 1 ml of culture medium at 54h of incubation to produce an initial concentration of 10^{-3} M.

The sulphonamides, acetazolamide, ethoxzolamide and benzolamide were dissolved in SSS (10^{-2} M, or appropriate dilutions) and 10 μl was added to SEF at 54h of incubation to produce nominal concentrations of 10^{-3} to 10^{-6} M, assuming the volume of SEF to be 100 μl . For each sulphonamide, dissolved in SSS at 2×10^{-2} M, 50 μl was added at 54 h of incubation to 1 ml of culture medium to produce a concentration of 10^{-3} M. Control cultures had the appropriate volume of SSS added to culture medium or SEF at 54h of incubation. All inhibitors were purchased from Sigma, except benzolamide, a gift of Dr T.H. Maren.

2 ***Sampling protocol***

At 54h of incubation under sterile conditions, amaranth dye addition followed by SEF removal
4 for volume estimation was performed first, then sampling of SEF and culture medium for sodium
concentration and osmolality measurement, and finally addition of carrier or inhibitor. At 72h of
6 incubation a sample of SEF for volume estimation was removed first, followed by sampling of
fluid and culture medium for measurement of sodium concentration and osmolality.

8 ***Statistical analysis***

The effect of inhibitors of ion transport upon SEF volume, sodium concentration and osmolality
10 were compared by one-way analyses of variance. Individual means were compared by Student-
Newman-Keuls procedure or by an independent two-sample *t*-test (Sokal and Rohlf, 1969). All
12 reported values represent the mean \pm standard error and the level of significance was taken as
 $P < 0.05$. All statistics were conducted using Minitab v.11 (Minitab Users Guide, 1993).

14 ***Carbonic anhydrase histochemistry***

Fixation and embedding

16 Seven blastoderms were cultured as above and after 54 h of incubation three of these cultures
were harvested for histochemical staining, the four remaining had carrier added to the SEF and
18 were harvested at 72h of incubation. Each blastoderm was submerged in Pannett & Compton's
(1924) Ringer at 22.0 ± 0.5 °C, the blastoderm was floated free and cut down each side of the
20 embryo to produce two pieces of the extra-embryonic tissues. These were placed in 2-3 ml of
2.5 % glutaraldehyde in 0.067 M sodium cacodylate buffer (pH 7.4) at 4 °C (Ekstedt and
22 Ridderstrale, 1992), and left at this temperature for 45 minutes. The tissue pieces were washed
three times with double distilled water. Fixed tissue was embedded in a plastic resin (JB4, Agar
24 Scientific Ltd.). Dehydration was achieved using mixtures of water and JB4 solution A, the latter
containing 0.9 g of benzoyl peroxide/100 ml of JB4 solution A. Tissue pieces were shaken for
26 10 minutes at one cycle per second on a mechanical shaker (Janke and Kunkel, Type Vx8) in
50 % (1 part water to 1 part JB4 solution A), 75 % (1 part water to 3 parts JB4 solution A) and

90 % (1 part water to 9 parts JB4 solution A) (Ekstedt and Ridderstrale, 1992) . Finally, the
blastoderm pieces were shaken for 1 hour with 4 changes of undiluted JB4 solution A. Final
resin mixture was prepared by adding 1 ml of JB4 solution B to the 25 ml of degassed JB4
solution A. The resin mixture was pipetted into polyethylene BEEM flat embedding moulds
(Agar Scientific Ltd.), one blastoderm piece placed within each mould, and the resin left to
polymerise for 2-3 hours. Resin blocks were stored at -20 °C for a maximum of two weeks and
then used for sectioning.

Staining

The method used was Ridderstrale's (1976) modification of Hansson's (1968) cobalt sulphide
precipitation method. JB4 blocks were cut dry at 2 μ sections on a Reichert-Jung Ultracut using
glass knives. Sections were floated and expanded on a large drop of distilled water on individual
coverslips. All changes of staining solutions took place on the original coverslip and throughout
the sections were floated on the liquid surface. Alternate serial sections were assessed for non-
specific staining by incubation with 10^{-5} M acetazolamide solution in double distilled water for 30
minutes 22.0 ± 0.5 °C (Maren, 1980; Holm *et al.*, 1996). All sections were then incubated for 15
minutes with a medium consisting of 17 ml of 1 part 0.2 M CoSO_4 , 6 parts 0.5 M H_2SO_4 , and 10
parts 1/15 M KH_2PO_4 mixed with 40 ml of 18.75 g L^{-1} NaHCO_3 , all dissolved in double distilled
water and containing 10^{-5} M acetazolamide for those sections that had been pre-incubated with
acetazolamide. Sections were then rinsed three times with a phosphate buffer, 6.7×10^{-4} M, pH
5.9, in double distilled water, and then blackened for 5 minutes with freshly prepared 0.5 %
 $(\text{NH}_4)_2\text{S}$ in double distilled water. Sections were then rinsed with three changes of distilled
water, air-dried and mounted with Eukitt (Agar Scientific Ltd.) and photographed using a Nikon
Labophot-2 (Kingston-upon-Thames, UK) with bright-field illumination. The image was output to
a digital camera (Leica DC-200, 2.6 megapixels with 24 bit colour depth; Leica Microsystems,
Milton Keynes, UK) and images were saved into Adobe Photoshop v. 5.0. using a Dell Optiplex
GXa 300 MHz.

2 RESULTS

4 *Effects of inhibitors of ion transport*

6 **Rate of production of sub-embryonic fluid**

8 Of the inhibitors of ion transport added to SEF only ouabain decreased J_{ap} as the concentration
 10 added to the SEF was increased (one-way anova: ouabain, $F_{3,20} = 560.61$, $P < 0.001$), and J_{ap}
 12 was lower than corresponding controls (Figure 1). All means differed from each other (Figure 1).
 14 When compared with cultures with carrier added the highest concentration of ouabain applied to
 the SEF, 10^{-3} M, decreased J_{ap} by a factor of one hundred (Figure 1). For cultures where 10^{-3} M
 ouabain was added to the culture medium, J_{ap} was not different those cultures with carrier
 added (10^{-3} M in culture medium $11.18 \pm 0.01 \mu\text{l h}^{-1}$, carrier $11.19 \pm 0.02 \mu\text{l h}^{-1}$, $t_{10} = 1.10$, $P = 0.30$)
 and were much higher than J_{ap} for the lowest concentration of ouabain (10^{-6} M) in the SEF ($t_{10} =$
 326.97 , $P < 0.001$ for J_{ap} ; Figure 1).

16
 Amiloride also reduced J_{ap} as the concentration increased but only when added to the culture
 18 medium (one-way anova, $F_{3,20} = 1000.00$, $P < 0.001$) and all means differed from each other
 (Figure 1). J_{ap} was lower than all corresponding controls and J_{ap} was decreased by a factor of
 20 approximately ten at the highest concentration of amiloride (Figure 1). In contrast, amiloride at
 10^{-3} M added to SEF produced no change in J_{ap} compared with its control (10^{-3} M amiloride in
 22 SEF $11.12 \pm 0.02 \mu\text{l h}^{-1}$, carrier $11.17 \pm 0.01 \mu\text{l h}^{-1}$, $t_{10} = 1.98$, $P = 0.08$). Also when 10^{-6} M amiloride
 was added to the culture medium, J_{ap} was lower than that for 10^{-3} M amiloride added to the SEF
 24 ($t_{10} = 30.88$, $P < 0.001$).

26 NEM added to the medium also decreased J_{ap} (one-way anova, $F_{3,20} = 1203.37$, $P < 0.001$), but
 only at the highest concentration (10^{-3} M) was J_{ap} reduced significantly and was 89% of its
 28 control (Figure 1). With 10^{-3} M NEM added to SEF J_{ap} did not differ from that for cultures with
 carrier (10^{-3} M NEM in SEF $11.23 \pm 0.02 \mu\text{l h}^{-1}$, carrier $11.23 \pm 0.02 \mu\text{l h}^{-1}$, $t_{10} = 0.13$, $P = 0.90$).

Hence, J_{ap} of cultures with 10^{-3} M NEM added to the SEF exceeded that for cultures with the same concentration of NEM applied to the culture medium ($t_{10} = 44.55$, $P < 0.001$).

Neither SITS nor DIDS affected J_{ap} at any concentration when added to the SEF (one-way anova: SITS, $F_{3,20} = 0.17$, $P = 0.91$; DIDS, $F_{3,20} = 0.46$, $P = 0.72$). For SITS at 10^{-3} M in SEF J_{ap} was $11.24 \pm 0.01 \mu\text{l h}^{-1}$ compared with control of $11.24 \pm 0.01 \mu\text{l h}^{-1}$. For DIDS at 10^{-3} M in SEF J_{ap} was $11.23 \pm 0.01 \mu\text{l h}^{-1}$ compared with control of $11.24 \pm 0.01 \mu\text{l h}^{-1}$. Additionally, for 10^{-3} M SITS added to the culture medium, J_{ap} did not differ from corresponding cultures with carrier added: with 10^{-3} M in culture medium J_{ap} was $11.24 \pm 0.01 \mu\text{l h}^{-1}$ and for carrier $11.24 \pm 0.01 \mu\text{l h}^{-1}$ ($t_{10} = 0.56$, $P = 0.59$). Likewise, for 10^{-3} M DIDS added to the culture medium, J_{ap} was not different from control cultures: with 10^{-3} M DIDS in culture medium J_{ap} was $11.24 \pm 0.01 \mu\text{l h}^{-1}$ and for carrier $11.23 \pm 0.01 \mu\text{l h}^{-1}$ ($t_{10} = 0.37$, $P = 0.72$).

SEF sodium concentration and osmolality

For control cultures, and for cultures measured at 54 h, mean [Na] of SEF for treated cultures exceeded that of the corresponding medium by a small but consistent amount (about 2 mM).

For example, at 54h of incubation in control cultures for 10^{-3} M ouabain added to the SEF the mean [Na] of SEF was greater than that of the culture medium by more than 2.0 mM (Table 1 and culture medium 69.33 ± 0.33 mM, $t_{10} = 5.92$, $P = 0.004$); for control cultures at 54h with 10^{-3} M amiloride in the medium, SEF [Na] exceeded the medium by 1.7 mM (Table 2 and culture medium 69.17 ± 0.17 mM, $t_{10} = 7.07$, $P < 0.001$); and for control cultures for 10^{-3} M NEM in medium at 54 h of incubation, mean SEF [Na] for was greater than that of the medium by almost 2 mM (Table 2 and culture medium 69.33 ± 0.21 mM, $t_{10} = 6.82$, $P = 0.001$).

Whilst at 54h of incubation in those cultures with 10^{-3} M ouabain added to the SEF the mean [Na] did not differ between treated and control cultures (Table 1), by 72 h of incubation those cultures with 10^{-3} M ouabain added to SEF had a mean [Na] that was about 2 mM lower than corresponding control (Table 1). In contrast, however, [Na] of SEF for cultures with 10^{-3} M

ouabain added to the culture medium did not differ from controls at 72h of incubation (Table 1, italic). Both amiloride and NEM decreased SEF [Na] only when added to the medium. By 72 h of incubation for both concentrations of amiloride added to the medium the mean [Na] of SEF had decreased by about 1 mM compared to controls (Table 2), whereas with 10^{-3} M NEM added to the medium the mean [Na] of SEF was 1.84 mM lower than the control (Table 2), but there was no change in [Na] of SEF for cultures with 10^{-6} M NEM added to the culture medium. For cultures with 10^{-3} M amiloride or NEM added to the SEF the mean [Na] of SEF did not differ from controls (Table 2, italic).

In cultures with ouabain added to SEF the osmolality of SEF and medium was very similar, for example at 54h of incubation SEF osmolality of 10^{-3} M ouabain controls did not differ from the medium osmolality of 243.67 ± 0.21 mOsm Kg⁻¹. Also osmolality of SEF from cultures with ouabain added to the medium did not differ from any corresponding controls. (Table 1). The mean osmolality of SEF from cultures with amiloride or NEM added to the medium did not differ from any corresponding controls (Table 2) and osmolality was very similar in medium and SEF: for example, at 54 h of incubation mean SEF osmolality (Table 2) of controls did not differ from medium osmolality of 244.00 ± 0.26 mOsm Kg⁻¹ (n=6), and at 72h of incubation control cultures for 10^{-3} M NEM in the medium the mean osmolality of SEF and medium were both 243.50 ± 0.22 mOsm Kg⁻¹ (n=6). Also the osmolality of SEF in cultures with amiloride or NEM added to SEF were very little different from controls (Table 2). Both SITS and DIDS, whether applied in the SEF or to the culture medium had no effect on SEF [Na] or osmolality.

22

Effects of carbonic anhydrase inhibitors

24

Rate of production of sub-embryonic fluid

When added to the SEF sulphonamides decreased J_{ap} as concentration increased (Figure 2; one-way anova: ethoxzolamide, $F_{3,20} = 7600.22$, $P < 0.001$; acetazolamide, $F_{3,20} = 5826.50$, $P < 0.001$; benzolamide, $F_{3,20} = 3320.58$, $P < 0.001$) and J_{ap} was lower than corresponding controls with carrier added (Figure 2). At 10^{-3} M, J_{ap} for ethoxzolamide-treated cultures was reduced by

28

approximately a half compared with the control cultures, whereas for acetazolamide-treated cultures J_{ap} was reduced to about one third, and for benzolamide J_{ap} was decreased by a factor of ten. At 10^{-6} M benzolamide had reduced J_{ap} to about the same level as 10^{-3} M ethoxzolamide (Figure 2), although even at 10^{-6} M ethoxzolamide produced a small, but significant, decrease in J_{ap} compared with its control ($t_{10} = 6.84$, $P=0.001$).

However, sulphonamides added to culture medium had no affect on J_{ap} . For 10^{-3} M ethoxzolamide added to the culture medium, J_{ap} did not differ from corresponding control cultures (in culture medium J_{ap} $11.03\pm 0.01 \mu\text{l h}^{-1}$, for carrier J_{ap} $11.03\pm 0.01 \mu\text{l h}^{-1}$, $t_{10} = 0.48$, $P = 0.64$). Consequently, for cultures with 10^{-3} M ethoxzolamide added to the medium, J_{ap} was greater than the mean for 10^{-6} M ethoxzolamide added to SEF ($t_{10} = 2.74$, $P = 0.021$; Figure 2). Likewise, for those cultures with 10^{-3} M acetazolamide added to the culture medium J_{ap} did not differ from corresponding cultures with carrier added (in culture medium, J_{ap} $11.04\pm 0.01 \mu\text{l h}^{-1}$, carrier $11.04\pm 0.01 \mu\text{l h}^{-1}$, $t_{10} = 0.01$, $P = 0.99$) and for these cultures J_{ap} was greater than for J_{ap} at 10^{-6} M acetazolamide added to SEF ($t_{10} = 13.83$, $P < 0.001$; Figure 2). For 10^{-3} M benzolamide added to the culture medium, J_{ap} did not differ from corresponding controls (in culture medium J_{ap} $11.14\pm 0.03 \mu\text{l h}^{-1}$, carrier J_{ap} $11.14\pm 0.02 \mu\text{l h}^{-1}$, $t_{10} = 0.16$, $P = 0.87$). The mean J_{ap} for 10^{-6} M benzolamide added to SEF was substantially less than mean J_{ap} for cultures with 10^{-3} M benzolamide in culture medium ($t_{10}=124.90$, $P < 0.001$; Figure 2).

SEF sodium concentration and osmolality

For control cultures, or cultures before addition of sulphonamides to the SEF, [Na] of SEF always exceeded that of the medium by a small but consistent amount (~2 mM). For example, at 54 h of incubation for 10^{-3} M ethoxzolamide cultures the mean [Na] for controls was greater than that of the medium by 2.00 mM (Table 3 and culture medium 69.17 ± 0.17 mM, $t_{10} = 8.49$, $P < 0.001$), for 10^{-6} M acetazolamide cultures the mean [Na] for controls was greater than the medium by 1.33 mM (Table 3 and medium culture 69.00 ± 0.10 mM, $t_{10} = 10.95$, $P = 0.001$) and for 10^{-3} M benzolamide cultures at 72 h of incubation the mean [Na] for controls at was greater

than that of the medium by 3.00 mM (Table 3 and culture medium 68.50 ± 0.22 mM, $t_{10} = 9.50$, $P < 0.001$).

However, although at 54 h of incubation for all sulphonamides the mean [Na] of SEF of treated cultures did not differ from the controls (Table 3), by 72 h of incubation SEF from cultures with sulphonamides added to the SEF had a mean [Na] which was about 2 mM lower than controls (Table 3). In contrast, the mean [Na] of SEF from cultures with 10^{-3} M sulphonamides added to the culture medium was no different from that in controls (Table 3, italic) and greater than that for cultures in which 10^{-3} M ethoxzolamide was added to the SEF (Table 3).

10

In cultures with sulphonamides added to SEF the mean osmolality of SEF did not differ from that of the controls (Table 3). In addition, the mean osmolality of SEF and culture medium did not differ: for example, for 10^{-3} M ethoxzolamide control cultures after 72 h of incubation mean SEF osmolality (Table 3) did not differ from the medium osmolality, 242.33 ± 0.21 mOsm Kg⁻¹ (n=6); for 10^{-3} M acetazolamide at 72h mean SEF osmolality for the control cultures (Table 3) was no different from the medium osmolality, 244.83 ± 0.31 mOsm Kg⁻¹ (n=6); and for 10^{-3} M benzolamide at 72h mean SEF osmolality for the control cultures (Table 3) was no different from the medium osmolality, 244.33 ± 0.21 mOsm Kg⁻¹ (n=6).

20 ***Localisation of carbonic anhydrase activity***

22 At both 54h and 72h of incubation the *area vasculosa* of cultured blastoderms was covered with endodermal cells on the side facing the SEF. These cells were largely columnar: their apical surface contacted the mesodermal vasculature, itself covered by an epithelium, and their basal surface contacted the SEF. Staining for carbonic anhydrase activity was distinguishable as black cobalt sulphide precipitate in those sections to which no acetazolamide was added to the incubation medium. For sections from cultured blastoderms at 54 h of incubation that had been stained for carbonic anhydrase activity in the absence of 10^{-5} M acetazolamide, cobalt precipitation was predominantly located at the intercellular junctions between endodermal cells

(Figure 3A), and, when present, extended for the whole length of these junctions (arrows, Figure 3A, B). In many instances this cobalt precipitation had a characteristic parallel line appearance, as can be seen for the two arrowed examples in Figure 3B. However, not all cells exhibited staining for carbonic anhydrase activity. At this stage of incubation the endodermal cells were usually filled with cytoplasm and contained granular inclusions, presumably yolk granules (Figure 3B), even in those cells with staining for carbonic anhydrase at the intercellular junctions (Figure 3B).

At 72 h of incubation, staining for carbonic anhydrase activity, in the absence of 10^{-5} M acetazolamide, was found at most of the intercellular junctions of endodermal cells beneath the vasculature (Figure 3C). Staining for carbonic anhydrase activity was never observed associated with the basal plasma membrane. These endodermal cells were vacuolated (Figure 3C, v), often with little cytoplasm visible. Cobalt precipitation was absent at the intercellular junctions in the presence of 10^{-5} M acetazolamide (for example adjacent sections Figure 3C and D, arrows, endodermal cells X, Y and Z), and although some cobalt precipitation was discernible near the apical membrane of the endodermal cells in sections incubated with 10^{-5} M acetazolamide, there was no evidence of substantial staining for carbonic anhydrase activity in the cytoplasm (e.g. Figure 3C and D, cell X). At this period of incubation the endodermal cells again exhibited a parallel line structure of cobalt precipitation at the intercellular junction (Figure 3E).

DISCUSSION

This study clearly demonstrates that fluid transport by the blastoderm of Japanese quail in culture is crucially dependent upon sodium transport into the SEF. Of the inhibitors of ion transport tested, ouabain, an inhibitor of Na^+/K^+ ATPase, reduced fluid production only when added to the SEF and did so substantially, even at the lowest dose tested. From this observation, we conclude that a Na^+/K^+ ATPase is resident on the plasma membrane of cells facing the SEF. Previously, a Na^+/K^+ ATPase had been reported from the epiblast of the avian

blastoderm, but at an earlier stage of development (16-20h chick embryo, Stern and Mackenzie, 1983; Stern, 1991). Amiloride, a known inhibitor of passive Na^+/H^+ exchange (Benos, 1982), also has been shown to substantially reduce the volume of SEF produced by the quail blastoderm in culture, but only when added to the culture medium (Babiker and Baggott, 1995). As amiloride was effective in reducing the rate of SEF production in our study, and again only when applied to the culture medium, we suggest that *in situ* a sodium ion/hydrogen ion exchanger is located on a plasma membrane facing the albumen. We propose, therefore, that the accumulation of sodium in the SEF at concentrations in excess of those in albumen (Howard, 1957; Babiker and Baggott, 1992,1995), is produced by the movement of sodium from albumen into the transporting cell, facilitated by a sodium ion/hydrogen ion exchanger on the albumen-facing side of the blastoderm, and then the exit of sodium via a Na^+/K^+ ATPase on the SEF-facing side of the blastoderm. This proposal is incorporated into the model for ion movements and fluid production shown in Figure 4. However, for such a transport process to operate there must be a source of protons for the sodium ion/hydrogen ion exchanger.

Carbonic anhydrase catalyses the hydration of carbon dioxide and the carbonic acid produced dissociates to produce protons and bicarbonate ions (Maren, 1967). All carbonic anhydrase inhibitors tested were effective in reducing SEF production, but only when added to the SEF. As benzamide had by far the greatest potency in reducing the rate of SEF production, and as of the three sulphonamides tested it has the least ability to penetrate plasma membranes (Maren, 1982; Wistrand and Knuutila, 1989), we suggest that fluid production is dependent primarily upon a membrane-associated isozyme of carbonic anhydrase. We attribute the lesser potency of acetazolamide and ethoxzolamide to, in effect, a lower concentration at the site of enzyme action due to their dilution within the tissues of the blastoderm. Previously, acetazolamide had been shown to produce a dose-related reduction in volume of SEF secreted by the quail blastoderm in culture, but only when added to SEF (Babiker and Baggott, 1995).

The histochemical localisation of carbonic anhydrase activity demonstrated an association between intense staining for carbonic anhydrase activity and the lateral plasma membranes of

the endodermal cells of the *area vasculosa* (Figure 3). This suggests that the carbonic
2 anhydrase isozyme of these cells is primarily the membrane-associated form, CA IV (Dodgson,
1991a), especially as the histochemistry indicated no substantive cytoplasmic carbonic
4 anhydrase activity (e.g. Figure 3C and D, cell X). We propose, therefore, that hydration of
carbon dioxide catalysed by carbonic anhydrase takes place in the endodermal cells and is the
6 source of protons for the sodium ion/hydrogen ion exchanger. It is likely, therefore, that the
sodium ion/hydrogen ion exchanger is located on the apical surface of the endoderm cells, as
8 suggested by Figure 4. Furthermore, the supply of protons from carbon dioxide for the apical
sodium ion/hydrogen ion exchanger would furnish cytosolic sodium to a Na⁺/K⁺ ATPase located
10 on the basolateral membrane of the endodermal cell, and so ensure the transport of sodium into
the SEF, as is indicated in Figure 4.

12
However, as the inhibitors of cellular bicarbonate and chloride exchange, SITS and DIDS
14 (Alpern and Chambers, 1987; Aronson, 1989; Greger *et al.*, 1990; Boron *et al.*, 1997), did not
alter fluid production, it would appear that exchangers of this type were not present in the
16 blastoderm; thus, the fate of bicarbonate ions produced by such carbon dioxide hydration
remains unresolved. In Figure 4 we propose that the endodermal cell possesses a membrane-
18 associated carbonic anhydrase enzyme with vectorial properties: bicarbonate ions would exit to
the SEF and protons be delivered into the cytosol. Two pieces of evidence support this
20 proposition. First, Babiker and Baggott (1991) found that *in ovo* the bicarbonate ion
concentration of SEF increased between 60 and 72 h of incubation, and second Diaz *et al*
22 (1982) have reported the transport of protons and bicarbonate ions in opposite directions by CA
IV inserted in an artificial bilayer.

24
In some aspects, fluid production and ion transport by the quail blastoderm resemble these
26 processes in the epithelial cells of the vertebrate gall-bladder, that utilise an apical sodium
ion/hydrogen ion exchanger (Reuss, 1989) and a membrane-associated carbonic anhydrase
28 (Persson and Larson, 1986; Parkkila *et al.*, 1996). Ion transport is less like that in the kidney
proximal tubule cell where CA IV is extracellular (Brown *et al.*, 1990; Dodgson, 1991b),

although these cells do use an apical sodium ion/hydrogen ion exchanger (Smith and Benos, 1991). However, unlike these tissues, cytoplasmic carbonic anhydrase activity was not detectable histochemically in the endodermal cells of the blastoderm, as was found in kidney tubule cells (Kumpulainen, 1984) or gall-bladder epithelial cells (Juvonen *et al.*, 1994). So whilst in endodermal cells protons are, apparently, supplied by the hydration of carbon dioxide catalysed by carbonic anhydrase, this supply does not appear to involve the co-operation of an intracellular carbonic anhydrase with an extracellular/membrane-associated isozyme, as found in other tissues (Sly and Hu, 1995).

Proton-motive V-ATPases are widely distributed in transporting epithelia (Wieczorek *et al.*, 1999) and are inhibitable by a number of specific agents, including N-ethylmaleimide (Ehrenfeld and Klein, 1997). The inhibition of SEF production by NEM only when added to the culture medium suggests that a V type H⁺ ATPase is located on the albumen-facing surface of the blastoderm. Although the magnitude of the inhibition by NEM was small, and only occurred at the highest dose applied, such an ATPase would assist in the movement of sodium into the endodermal cell if, as proposed in Figure 4, it was located on the apical surface: an outwardly-directed proton movement would produce an electrical gradient for the inward movement of sodium. Avian blastoderm is not, however, like the frog skin, where sodium transport is strongly dependent upon an apically located V type H⁺ ATPase and where carbonic anhydrase has a relatively minor role in the supply of protons during acid-base disturbances (Ehrenfeld and Klein, 1997); nor is it similar to the kidney tubule where V type H⁺ ATPase has an essential role in hydrogen ion secretion (Gluck *et al.*, 1996).

However, some other aspects of ion transport and fluid production by the blastoderm are unresolved. Babiker and Baggott (1995) found that chloride-depleted media reduced substantially fluid production by the blastoderm in culture, yet neither furosemide, an inhibitor of Na⁺/2Cl⁻/K⁺ cotransport (O'Grady *et al.*, 1990), nor DIDS altered fluid production. At least two alternatives remain to be explored: the movement of chloride by paracellular route and/or a transcellular route (Zeuthen, 1992).

2 The blastoderm possesses the histoarchitecture required for the production of local osmotic
gradients as the endodermal cells of the *area vasculosa* are separated by narrow, long and
4 tortuous lateral intercellular spaces (LIS, Figure 4) with tight junctions at their apical ends
(Mobbs and MacMillan, 1979). Although recent evidence has shown that tight junctions from a
6 variety of transporting epithelia are, in fact, leaky, lateral intercellular spaces of this type have
the capacity to produce local osmotic gradients (Spring, 1998). The fluid that emerges from the
8 lateral intercellular spaces may be either hypertonic or isotonic, depending upon geometry and
the water permeability of the plasma membranes (Diamond and Bossert, 1967). In control
10 blastoderms SEF had a greater concentration of sodium than the albumen culture medium, but
with both fluids of the same osmolality. These observations differ from those *in ovo*, where
12 osmolality, as well as sodium concentration, of SEF is reported to exceed that of the albumen
(Howard, 1957; Babiker and Baggott, 1992) . However, as *in ovo* albumen and SEF osmolality
14 were measured in bulk fluids, these values may have been unrepresentative of secreted fluid, in
contrast to cultures where both SEF and albumen medium were sampled adjacent to the
16 blastoderm. The actions of inhibitors of ion transport and sulphonamides upon SEF production
and composition were consistent with fluid generation by local osmotic gradients within the
18 lateral intercellular spaces (Babiker and Baggott, 1995). Whilst in those cultures where fluid
production was experimentally reduced there was no detectable change in SEF osmolality,
20 there was, as expected, a decrease in the sodium concentration of the fluid, as inhibition of any
of the constituent parts of the transport process would reduce sodium transport into the SEF.
22 Presumably, the osmolality of the SEF did not reflect the decrease in sodium concentration
because of changes in other osmotically active constituents of this fluid.

24
The evidence presented by this study suggests that in early incubation a primary function for the
26 endodermal cells of the *area vasculosa* is production of SEF, whereas previously the uptake of
yolk by these cells has been presumed to be their main role (Mobbs and MacMillan, 1981).
28 However, although the route of transepithelial water flow in epithelia with tight junctions is still in
dispute (Spring, 1998), some evidence indicates that transcellular water flow can occur: for

example, Persson and Larson (1986) found that when acetazolamide was used to inhibit fluid
2 absorption from gall-bladder there was an associated decrease in epithelial cell volume. In the
endodermal cells of the blastoderm, large intracellular vacuoles were observed after 72h of
4 incubation and they may have been fluid- filled, given that the tissues were embedded in a
water-based medium. Undoubtedly, however, further information on the properties and
6 distribution of endodermal cell types within the *area vasculosa* throughout the period SEF
production is required for both the Japanese quail and other avian species.

8
It is likely, therefore, that during early incubation the level of CO₂ at the endodermal cells is
10 important for the normal production of SEF. Although the increased tolerance to CO₂ exhibited
by the chick embryo over the first 8 days of incubation (Taylor *et al.*, 1956; Taylor and
12 Kreutziger, 1965) is consistent with this proposition, the effects of CO₂ during preincubation
storage upon embryonic viability are less supportive (Brake *et al.*, 1997). For example, turkey
14 eggs stored in bags with CO₂ exhibited decreased embryo viability (Becker *et al.*, 1968), but
chicken eggs stored for 14 days with CO₂ showed increased apparent fertility, decreased the
16 numbers of early dead embryos and a lower albumen pH (Walsh *et al.*, 1995). The storage of
eggs in air decreases the total CO₂ content and increases the pH of albumen (Cotterill *et al.*,
18 1958; Mueller, 1958), but the latter can be a poor indicator of albumen CO₂ content as an
albumen pH greater 9.3 encompasses a wide range of CO₂ content (Mueller, 1958). Albumen
20 may, therefore, be important as a source of CO₂ for the blastoderm endodermal cells, but the
amount available will depend upon the CO₂ buffering characteristics of the albumen as well as
22 the PCO₂ of the environment. Whilst, evidently CO₂ is essential for SEF production by the
blastoderm, and the production of this fluid is a pivotal event of early development (Deeming,
24 1989), if CO₂ were to be used to promote viability of stored or incubated eggs then the effects of
CO₂ sources, such as albumen, upon CO₂ levels at the blastoderm clearly need to be assessed.

26
In summary, we conclude that SEF production by the blastoderm is predominantly a function of
28 the endodermal cells of the *area vasculosa* and that carbon dioxide plays an essential role in
the production of this fluid. The evidence presented further supports the proposal of Babiker and

1 Baggott (1995) that SEF is produced by a local osmotic gradients. We further conclude that the
2 endodermal cells of the blastoderm possess a Na^+/K^+ ATPase resident on the plasma
membrane facing the SEF, a sodium ion/hydrogen ion exchanger on their apical surface and
4 have, associated with the lateral plasma membrane, a carbonic anhydrase that supplies protons
for Na^+/H^+ exchange by the sodium ion/hydrogen ion exchanger.

6

8 **ACKNOWLEDGEMENTS**

We wish to thank the Chair of the Department of Biology, Birkbeck College for provision of
10 facilities and Dr H. Ratnajothi for his husbandry of the quail stocks. We are most grateful for the
late Dr T. H. Maren for the gift of benzolamide, and to Dr T Arnett, Department of Anatomy,
12 University College, London for use of his photomicroscope.

14 **REFERENCES**

16 ALPERN, R.J. & CHAMBERS, M. (1987) Basolateral membrane $\text{Cl}^-/\text{HCO}_3^-$ exchange in the rat
proximal convoluted tubule: Na-dependent and independent modes. *Journal of General*
18 *Physiology*, **89**: 581-598.

20 AR, A. (1991) Egg water movements during incubation, in: TULLETT S.G. (Ed.) *Avian*
Incubation, Poultry Science Symposium, No. 20, pp. 157-173 (London, Butterworth-
22 Heinemann).

24 ARONSON, P.S. (1989) The renal proximal tubule: A model for diversity of anion exchangers
and stilbene-sensitive anion transporters. *Annual Review of Physiology*, **51**: 419-441.

26

BABIKER, E.M. & BAGGOTT, G.K. (1991) The acid-base status of the sub-embryonic fluid of
28 the Japanese quail, *Coturnix coturnix japonica*, in: TULLETT S.G. (Ed.) *Avian Incubation*,
Poultry Science Symposium, No. 20, p. 326 (London, Butterworth-Heinemann).

30

- 1 BABIKER, E.M. & BAGGOTT, G.K. (1992) Effect of turning upon the sub-embryonic fluid and
2 albumen of the egg of the Japanese quail. *British Poultry Science*, **33**: 973-991.
- 4 BABIKER, E.M. & BAGGOTT, G.K. (1995) The role of ion transport in the formation of sub-
embryonic fluid by the embryo of the Japanese quail. *British Poultry Science*, **36**: 371-383.
- 6
- 8 BECKER, W.A., SPENCER, J.V. & SWARTWOOD, J.L. (1968) Carbon dioxide during storage
of chicken and turkey eggs. *Poultry Science*, **47**: 251-258.
- 10 BENOS, D.J. (1982) Amiloride: a molecular probe of sodium transport in tissues and cells.
American Journal of Physiology, **242** (Cell Physiology 11): C131-C145.
- 12
- 14 BORON, W.F., FONG, P., HEDIGER, M.A., BOULPAEP, E.L. & ROMERO, M.F. (1997) The
electrogenic $\text{Na}^+/\text{HCO}_3^-$ cotransporter. *Wiener Klinische Wochenschrift*, **109**: 445-56.
- 16 BRAKE, J., WALSH, T.J., BENTON, C.E., PETITTE, J.N. MEIJERHOF, R. & PENALVA, G.
(1997) Egg handling and storage. *Poultry Science*, **76**: 144-151.
- 18
- 20 BROWN D., ZHU X.L. & SLY W.S. (1990) Localization of membrane-associated carbonic-
anhydrase type-IV in kidney epithelial-cells. Proceedings of the National Academy of Sciences
of the United States of America, **87**: 7457-7461.
- 22
- 24 COTTERILL, O.J., GARDNER, F.A., FUNK, E.M. & CUNNINGHAM, F.E. (1958) Relationship
between temperature and carbon dioxide loss from shell eggs. *Poultry Science*, **37**: 479-483.
- 26 DEEMING, D.C. (1989) Importance of sub-embryonic fluid and albumen in the embryo's
response to turning of the egg during incubation. *British Poultry Science*, **30**: 591-606.
- 28

- DIAMOND, J. (1979) Osmotic water flow in leaky epithelia. *Journal of Membrane Biology*, **51**:
2 195-216.
- 4 DIAMOND, J.M. & BOSSERT, W.H. (1967) Standing-gradient osmotic flow. A mechanism for
coupling of water and solute transport in epithelia. *Journal of General Physiology*, **50**: 2061-
6 2083.
- 8 DIAZ, E., SANDBLOM, J.P. & WISTRAND, P.J. (1982) Selectivity properties of channels
induced by a reconstituted membrane-bound carbonic anhydrase. *Acta Physiologica*
10 *Scandinavica*, **116**: 461-463.
- 12 DODGSON, S.J. (1991a) The carbonic anhydrases. Overview of their importance in cellular
physiology and in molecular genetics, in: DODGSON, S.J., TASHIAN, R.E., GROS, G. &
14 CARTER, N.D. (Eds) *The Carbonic Anhydrases*, pp. 3-14 (New York, Plenum Press).
- 16 DODGSON, S.J. (1991b) Carbonic anhydrases in the kidney, in: DODGSON, S.J., TASHIAN,
R.E., GROS, G. & CARTER, N.D. (Eds) *The Carbonic Anhydrases*, pp. 345-350 (New York,
18 Plenum Press).
- 20 EHRENFELD, J. & KLEIN, U. (1997) The key role of the H⁺ V-ATPase in acid-base balance and
Na⁺ transport processes in frog skin. *Journal of experimental Biology*, **200**: 247-256.
22
- EKSTEDT, E. & RIDDERSTRALE, Y. (1992) Histochemical localization of carbonic anhydrase
24 in the testis and epididymis of the rabbit. *Acta anatomica*, **143**: 258-264.
- 26 FRITSCHÉ, C., KLEINMAN, BAIN, J.L.W., HEINEN, R.R. & RILEY, D.A. (1991) Carbonic
anhydrase and proton secretion in turtle bladder mitochondrial-rich cells. *American Journal of*
28 *Physiology*, **260** : F-443-F458.

- 1 GREGER, R., KUNZELMANN, K. & GERLACH, L. (1990) Mechanisms of chloride transport in
2 secretory epithelia. *Annals of the New York Academy of Sciences*, **574**: 403-15.
- 4 GLUCK, S.L., UNDERHILL, D.M., IYORI, M., HOLLIDAY, L.S., KOSTROMINOVA, T.Y. & LEE,
6 B.S. (1996) Physiology and biochemistry of the kidney vacuolar H⁺-ATPase. *Annual Review of
6 Physiology*, **58**: 427-445.
- 8 HANSSON, H.P.J. (1968) Histochemical demonstration of carbonic anhydrase activity in some
10 epithelia noted for active transport. *Acta Physiologica Scandinavica*, **73**: 427-434.
- 12 HOLM, L., RIDDERSTRALE, Y. & KNUTSSON, P-G. (1996) Localisation of carbonic anhydrase
12 in the sperm storing regions of the domestic hen oviduct. *Acta Anatomica*, **156**: 253-260.
- 14 HOWARD, E. (1957) Ontogenetic changes in the freezing point and sodium and potassium
16 content of the subgerminal fluid and blood plasma of the chick embryo. *Journal of Cellular and
16 Comparative Physiology*, **50**: 451-470.
- 18 JUVONEN, T., PARKKILA, S., PARKKILA, A-K., NIEMELA, O., LAJUNEN, L.H.J.,
20 KAIRALUOMA, M.I. & PERAMAKI, P. (1994) High-activity carbonic anhydrase isoenzyme (CA
20 11) in human gallbladder epithelium. *Journal of Histochemistry and Cytochemistry*, **42**: 1393-
22 1397.
- 24 KUMPULAINEN, T. (1984) Immunohistochemical localisation of human carbonic anhydrase
24 isoenzymes. *Annals of the New York Academy of Sciences*, **429**: 359-368.
- 26 MAREN, T.H. (1967) Carbonic anhydrase: chemistry, physiology and inhibition. *Physiological
28 Reviews*, **47**: 595-781.

- 1 MAREN, T.H. (1980) Kinetics, equilibrium and inhibition in the Hansson histochemical
2 procedure for carbonic anhydrase: a validation of the method. *Histochemical Journal*, **12**: 183-
3 190.
- 4
- 5 MAREN, T.H. (1982) Benzolamide. A renal carbonic anhydrase inhibitor, in: KARCH, F.E. (Ed.)
6 *Orphan drugs*, pp. 89-115 (New York, Dekker).
- 7
- 8 MAREN, T.H. (1988) The kinetics of HCO_3^- synthesis related to fluid secretion, pH control, and
9 CO_2 elimination. *Annual Review of Physiology*, **50**: 695-717.
- 10
- 11 MINITAB USERS GUIDE (1993) (Minitab. Inc.).
- 12
- 13 MOBBS, I. G. & MCMILLAN, D. B. (1979) Structure of the endodermal epithelium of the chick
14 yolk sac during early stages of development. *American Journal of Anatomy*, **155**: 287-309.
- 15
- 16 MOBBS, I. G. & MCMILLAN, D. B. (1981) Transport across endodermal cells of the chick yolk-
17 sac during early stages of development. *American Journal of Anatomy*, **160**: 285-308.
- 18
- 19 MUELLER, W.J. (1958) Shell porosity of chicken eggs. 1. CO_2 loss and CO_2 content of infertile
20 eggs during storage. *Poultry Science*, **37**: 437-444.
- 21
- 22 NEW, D.A.T. (1955) A new technique for the cultivation of the chick embryo in vitro. *Journal of*
23 *Embryology and Experimental Morphology*, **3**: 326-331.
- 24
- 25 NEW, D.A.T. (1956) The formation of sub-blastodermic fluid in hens' eggs. *Journal of*
26 *Embryology and Experimental Morphology*, **4**: 221-227.
- 27
- 28 O'GRADY S.M., MUSCH, M.W. & FIELD, M. (1990) Diuretic compounds structurally related to
furosemide. *Methods in Enzymology*, **191**: 781-792.

- 2 PANNETT, C.A. & COMPTON, A. (1924) The cultivation of tissues in saline embryonic juices.
Lancet, **206**: 381-384.
- 4
- 6 PARKKILA, S., PARKKILA, A-K., JUVONEN, T., WAHEED, A., SLY, W.S., SAARNIO, J.,
KAUNISTO, K., KELLOKUMPU, S. & RAJANIEMI, H. (1996) Membrane-bound carbonic
anhydrase IV is expressed in the luminal plasma membrane of the human gallbladder.
8 *Hepatology*, **24**: 1104-1108.
- 10 PERSSON, B-E. & LARSON, M. (1986) Carbonic anhydrase inhibition and cell volume
regulation in *Necturus* gallbladder. *Acta Physiologica Scandinavica*, **128**: 501-507.
- 12
- 14 REUSS, L. (1989) Ion transport across the gallbladder epithelium. *Physiological Reviews*, **69**:
503-545.
- 16 ROMANOFF, A.L. (1960) *The Avian Embryo* (New York, MacMillan).
- 18 ROMANOFF, A.L. (1967) *Biochemistry of the Avian Embryo* (New York, John Wiley).
- 20 RIDDERSTRALE, Y. (1976) Intracellular localisation of carbonic anhydrase in the frog nephron.
Acta Physiologica Scandinavica, **98**: 465-469.
- 22
- 24 SIMKISS, K. (1980) Water and ionic fluxes inside the egg. *American Zoologist*, **20**: 385-393.
- 26 SMITH, P.R. & BENOS, D.J. (1991) Epithelial Na⁺ channels. *Annual Review of Physiology*, **53**:
509-530.
- 28 SOKAL, R.R. & ROHLF, F.J. (1969) *Biometry*, 1st edition (San Francisco, W.H. Freeman).

- 1 SLY, W.S. & HU, P.Y. (1995) Human carbonic anhydrases and carbonic anhydrase
2 deficiencies. *Annual Review of Biochemistry*, **64**: 375-401.
- 4 SPRING, K.R. (1998) Routes and Mechanism of fluid transport by Epithelia. *Annual Review of
5 Physiology*, **60**: 105-119.
- 6
7 STERN, C.D. (1991) The sub-embryonic fluid of the egg of the domestic fowl and its
8 relationship to the early development of the embryo in: TULLETT S.G. (Ed.) *Avian Incubation*,
9 Poultry Science Symposium, No. 20, pp. 81-90 (London, Butterworth-Heinemann).
- 10
11 STERN, C.D. & MACKENZIE, D.O. (1983) Sodium transport and the control of epiblast polarity
12 in the early chick embryo. *Journal of Embryology and Experimental Morphology*, **77**: 73-98.
- 14 TAYLOR, L.W. & KREUTZIGER, G.O. (1965) The gaseous environment of the chick embryo in
15 relation to its development and hatchability. 2. Effect of carbon dioxide and oxygen levels during
16 the period of the fifth through the eighth days of incubation. *Poultry Science*, **54**: 98-106.
- 18 TAYLOR, L.W., SJODIN, R.A. & GUNNS, C.A. (1956) The gaseous environment of the chick
19 embryo in relation to its development and hatchability. 1. Effect of carbon dioxide and oxygen
20 levels during the first four days of incubation upon hatchability. *Poultry Science*, **35**: 1206-1215.
- 22 WALSH, T.J., RIZK, R.E. & BRAKE, J. (1995) Effects of temperature and carbon dioxide on
23 albumen characteristics, weight loss, and early embryonic mortality of long stored hatching
24 eggs. *Poultry Science*, **74**: 1403-1410.
- 26 WIECZOREK, H., BROWN, D., GRINSTEIN, S., EHRENFELD, J. & HARVEY, W.R. (1999)
27 Animal plasma membrane energization by proton-motive V-ATPases. *Bioessays*, **21**: 637-648.
- 28

- WISTRAND, P. J. & KNUUTTILA, K. G. (1989) Renal Membrane-Bound Carbonic-Anhydrase -
2 Purification and Properties. *Kidney International*, **35**: 851-859.
- 4 ZEUTHEN, T. (1992) From contractile vacuole to leaky epithelia. Coupling between salt and
water fluxes in biological membranes. *Biochimica et Biophysica Acta*, **1113**: 229-258.
- 6

Table 1. Mean values (\pm SEM) of concentration of sodium (Na, mM L⁻¹) and osmolality (Osm, mOsm Kg⁻¹) at 54h and 72h of incubation in sub-embryonic fluid (SEF) for cultures used as controls (C) and cultures treated (T) at 54h with ouabain, SITS, or DIDS at nominal concentrations of 10⁻³ M or 10⁻⁶ M in the SEF, or these inhibitors added at 10⁻³ M to the culture medium (in italics).

Inhibitor concentration	10 ⁻³ M in SEF		10 ⁻⁶ M in SEF		10 ⁻³ M in medium	
	54 h	72 h	54 h	72 h	54 h	72 h
<i>ouabain</i>						
Na (T)	71.33 \pm 0.21	69.33 \pm 0.21 ^{aA}	71.50 \pm 0.23	69.50 \pm 0.23 ^c	<i>71.50 \pm 0.23</i>	<i>71.50 \pm 0.23^B</i>
(C)	71.17 \pm 0.17	71.67 \pm 0.21 ^b	71.50 \pm 0.23	71.67 \pm 0.21 ^d	<i>71.17 \pm 0.17</i>	<i>71.50 \pm 0.23</i>
Osm (T)	243.67 \pm 0.21	243.83 \pm 0.17	243.67 \pm 0.21	243.83 \pm 0.17	<i>243.67 \pm 0.21</i>	<i>243.67 \pm 0.21</i>
(C)	243.67 \pm 0.21	243.67 \pm 0.21	243.67 \pm 0.21	243.67 \pm 0.21	<i>243.67 \pm 0.21</i>	<i>243.67 \pm 0.21</i>
<i>SITS</i>						
Na (T)	71.33 \pm 0.21	71.50 \pm 0.23	71.33 \pm 0.21	71.33 \pm 0.21	<i>71.33 \pm 0.21</i>	<i>71.50 \pm 0.23</i>
(C)	71.33 \pm 0.21	71.50 \pm 0.23	71.33 \pm 0.21	71.50 \pm 0.23	<i>71.33 \pm 0.21</i>	<i>71.50 \pm 0.23</i>
Osm (T)	243.33 \pm 0.21	244.33 \pm 0.21	243.33 \pm 0.21	244.33 \pm 0.21	<i>243.33 \pm 0.21</i>	<i>243.50 \pm 0.22</i>
(C)	243.33 \pm 0.21	243.50 \pm 0.22	243.33 \pm 0.21	243.50 \pm 0.22	<i>243.33 \pm 0.21</i>	<i>243.50 \pm 0.22</i>
<i>DIDS</i>						
Na (T)	71.17 \pm 0.17	71.50 \pm 0.23	71.17 \pm 0.17	71.50 \pm 0.23	<i>71.50 \pm 0.23</i>	<i>71.67 \pm 0.21</i>
(C)	71.33 \pm 0.21	71.50 \pm 0.23	71.33 \pm 0.21	71.50 \pm 0.23	<i>71.50 \pm 0.23</i>	<i>71.50 \pm 0.23</i>
Osm (T)	243.33 \pm 0.21	243.50 \pm 0.22	243.33 \pm 0.21	243.67 \pm 0.21	<i>243.33 \pm 0.21</i>	<i>243.50 \pm 0.22</i>
(C)	243.33 \pm 0.21	243.67 \pm 0.21	243.33 \pm 0.21	243.67 \pm 0.21	<i>243.33 \pm 0.21</i>	<i>243.67 \pm 0.21</i>

Sample sizes were 6 observations for treatments and 6 observations for control with carrier added. For each measure in the same column means with different lower case superscripts differ at $P < 0.001$. Within a row means with differing uppercase superscripts differ at $P < 0.001$.

2 **Table 2 .** Mean values (\pm SEM) for concentration of sodium (Na, mM L⁻¹) and osmolality (Osm,
 4 mOsm Kg⁻¹) at 54h and 72h of incubation in sub-embryonic fluid (SEF) for cultures used as
 controls (C) and cultures treated (T) at 54h with amiloride or NEM at initial concentrations of 10⁻³
 6 M or 10⁻⁶ M in the culture medium, or these inhibitors added at 10⁻³ M (nominal) to the SEF (in
 italics).

8

Inhibitor concentration	10 ⁻³ M in medium		10 ⁻⁶ M in medium		10 ⁻³ M in SEF	
	54 h	72 h	54 h	72 h	54 h	72 h
<i>amiloride</i>						
Na (T)	70.67 \pm 0.21	69.67 \pm 0.21 ^{aA}	70.67 \pm 0.21	69.97 \pm 0.21 ^c	70.67 \pm 0.21	71.00 \pm 0.26 ^B
(C)	70.83 \pm 0.17	71.17 \pm 0.17 ^b	70.83 \pm 0.17	71.17 \pm 0.17 ^d	70.83 \pm 0.17	71.00 \pm 0.26
Osm (T)	244.33 \pm 0.21	244.50 \pm 0.22	244.33 \pm 0.21	244.50 \pm 0.22	244.33 \pm 0.21	244.33 \pm 0.21
(C)	244.33 \pm 0.21	244.50 \pm 0.22	244.33 \pm 0.21	244.50 \pm 0.22	244.00 \pm 0.01	244.33 \pm 0.21
<i>NEM</i>						
Na (T)	71.33 \pm 0.21	69.33 \pm 0.21 ^{eC}	71.17 \pm 0.17	71.17 \pm 0.17	71.33 \pm 0.21	71.50 \pm 0.23 ^D
(C)	71.17 \pm 0.17	71.17 \pm 0.17 ^f	71.17 \pm 0.17	71.17 \pm 0.17	71.17 \pm 0.17	71.17 \pm 0.17
Osm (T)	243.33 \pm 0.21	243.50 \pm 0.22	243.33 \pm 0.21	243.50 \pm 0.22	243.33 \pm 0.21	243.50 \pm 0.22
(C)	243.50 \pm 0.22	243.50 \pm 0.22	243.50 \pm 0.22	243.50 \pm 0.22	243.50 \pm 0.22	243.50 \pm 0.22

10

Sample sizes were 6 observations for treatments and 6 observations for control with carrier
 12 added. For each measure in the same column means with different lower case superscripts
 differ at $P < 0.001$. Within a row means with differing uppercase superscripts differ at $P < 0.001$.

14

2 **Table 3.** Mean values (\pm SEM) of concentration of sodium (Na, mM L⁻¹) and osmolality (Osm,
 4 mOsm Kg⁻¹) at 54h and 72h of incubation in sub-embryonic fluid (SEF) from cultures used as
 controls (C) and cultures treated (T) at 54h with sulphonamides at nominal concentrations of 10⁻³
 6 M or 10⁻⁶ M in the SEF, or with 10⁻³ M added to the culture medium (in italics).

8

Sulphonamide concentration	10 ⁻³ M in SEF		10 ⁻⁶ M in SEF		10 ⁻³ M in medium	
	54 h	72 h	54 h	72 h	54 h	72 h
<i>Ethoxzolamide</i>						
Na (T)	71.17 \pm 0.17	69.17 \pm 0.17 ^{aA}	71.17 \pm 0.17	69.17 \pm 0.17 ^c	71.17 \pm 0.17	71.17 \pm 0.17 ^B
(C)	71.17 \pm 0.17	71.17 \pm 0.17 ^b	71.17 \pm 0.17	71.17 \pm 0.17 ^d	71.17 \pm 0.17	71.17 \pm 0.17
Osm (T)	242.17 \pm 0.17	242.50 \pm 0.22	242.17 \pm 0.21	242.33 \pm 0.21	242.17 \pm 0.17	242.33 \pm 0.21
(C)	242.17 \pm 0.22	242.33 \pm 0.17	242.17 \pm 0.22	242.33 \pm 0.17	242.17 \pm 0.17	242.33 \pm 0.21
<i>Acetazolamide</i>						
Na (T)	71.50 \pm 0.23	69.17 \pm 0.17 ^{eC}	71.67 \pm 0.21	69.33 \pm 0.21 ^g	71.33 \pm 0.21	71.33 \pm 0.21 ^D
(C)	71.50 \pm 0.23	71.50 \pm 0.23 ^f	71.33 \pm 0.21	71.33 \pm 0.21 ^h	71.50 \pm 0.23	71.50 \pm 0.23
Osm (T)	244.33 \pm 0.21	244.83 \pm 0.31	244.33 \pm 0.21	244.83 \pm 0.31	244.33 \pm 0.21	244.83 \pm 0.31
(C)	244.33 \pm 0.21	244.83 \pm 0.31	244.33 \pm 0.21	244.83 \pm 0.31	244.33 \pm 0.21	244.83 \pm 0.31
<i>Benzolamide</i>						
Na (T)	71.50 \pm 0.23	68.83 \pm 0.17 ^{iE}	71.50 \pm 0.23	68.83 \pm 0.17 ^k	71.50 \pm 0.34	71.67 \pm 0.21 ^F
(C)	71.50 \pm 0.23	71.50 \pm 0.23 ^j	71.50 \pm 0.23	71.50 \pm 0.23 ^l	71.50 \pm 0.34	71.67 \pm 0.33
Osm (T)	244.33 \pm 0.21	244.50 \pm 0.22	244.33 \pm 0.21	244.33 \pm 0.21	244.33 \pm 0.21	244.50 \pm 0.22
(C)	244.33 \pm 0.21	244.50 \pm 0.22	244.33 \pm 0.21	244.50 \pm 0.22	244.33 \pm 0.21	244.50 \pm 0.22

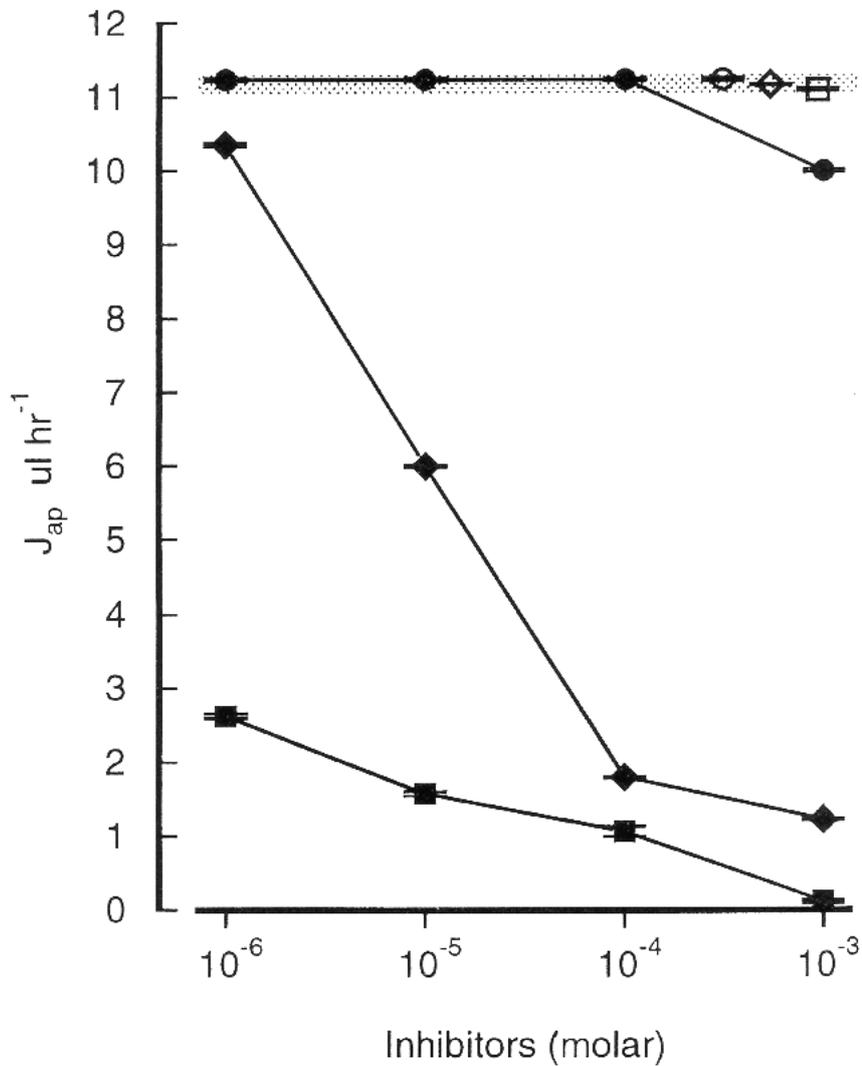
10 Sample sizes were 6 observations for treatments and 6 observations for control with carrier
 added. For each measure in the same column means with different lower case superscripts
 12 differ at $P < 0.001$. Within a row means with differing uppercase superscripts differ at $P < 0.001$.

14

16

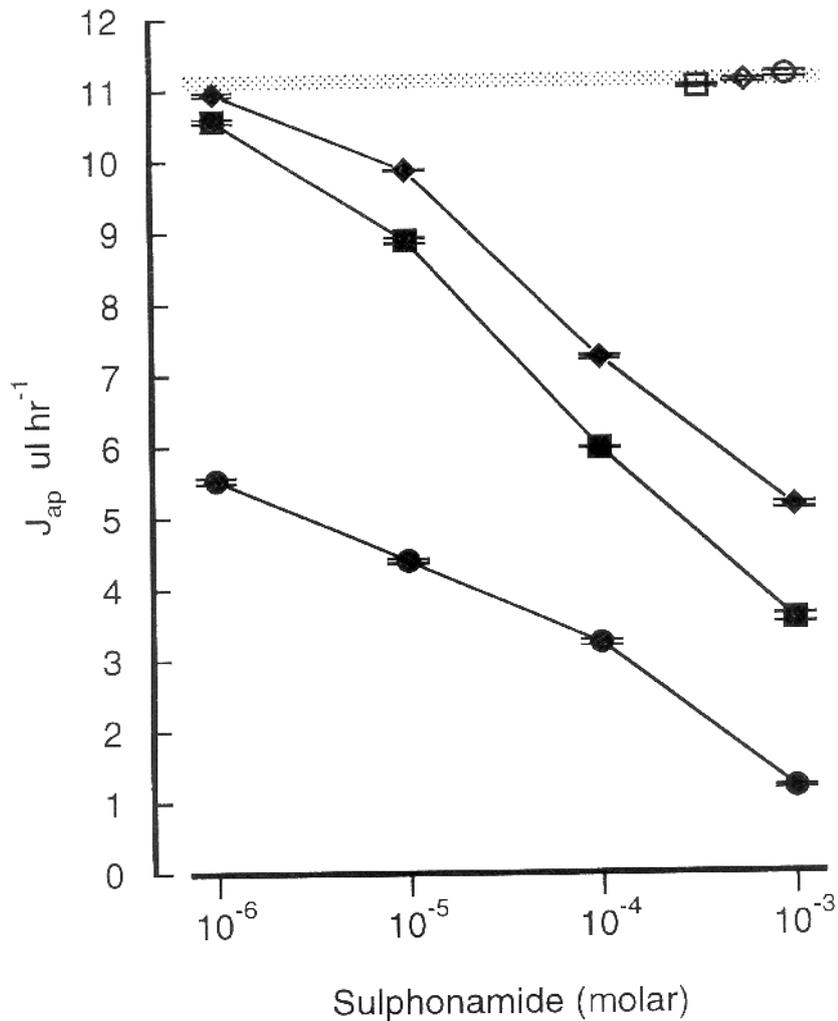
2 **Figure 1.** The effect of ouabain added to SEF (square symbol), of amiloride added to culture
 medium (diamond symbol), and NEM added to culture medium (circle symbol) at 54 h of
 4 incubation upon the mean (\pm SEM) rate of production of sub-embryonic fluid (J_{ap} $\mu\text{l h}^{-1}$) by the
 quail blastoderm in culture between 54 h and 72 h of incubation. Filled symbols represent J_{ap} in
 6 presence of inhibitor at concentrations indicated; for ouabain and amiloride all means differ from
 each other ($P < 0.05$). Open symbols are the mean values for controls, cultures with carrier
 8 added, for the 10^{-3}M cultures (symbols displaced). The stippled area encloses the range of
 mean values for all cultures with carrier added. Sample size is 6 for data points displayed.

10



2 **Figure 2.** The effect of the sulphonamides, ethoxzolamide (diamond symbol), acetazolamide
 (square symbol) and benzolamide (circle symbol) added to the sub-embryonic fluid at 54 h of
 4 incubation upon the mean (\pm SEM) rate of production of sub-embryonic fluid (J_{ap} $\mu\text{l h}^{-1}$) by the
 quail blastoderm in culture between 54 h and 72 h of incubation. Filled symbols represent J_{ap} in
 6 presence of sulphonamide at concentrations indicated; for each sulphonamide all means differ
 from each other ($P < 0.05$). Open symbols are the mean values for controls, cultures with carrier
 8 added, for the 10^{-3} M cultures (symbols displaced). The stippled area encloses the range of
 mean values for all cultures with carrier added. Sample size is 6 for data points displayed.

10



2 **Figure 3.** Localisation of carbonic anhydrase activity in *area vasculosa* endodermal cells.

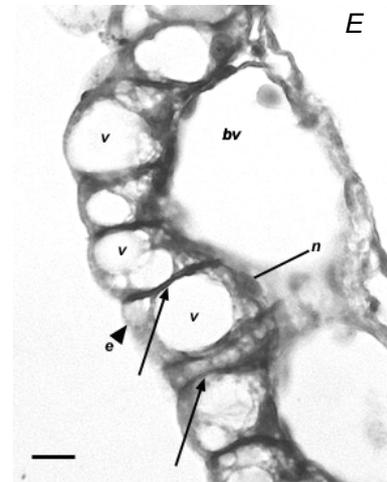
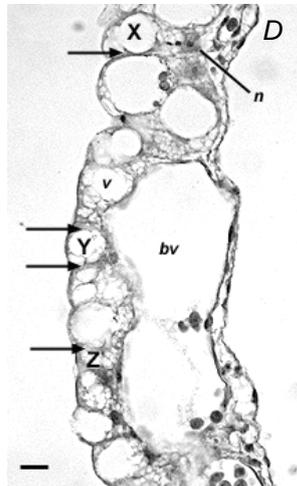
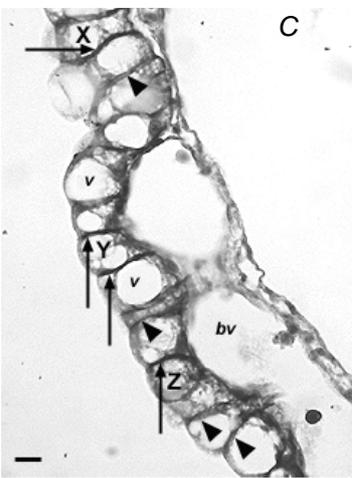
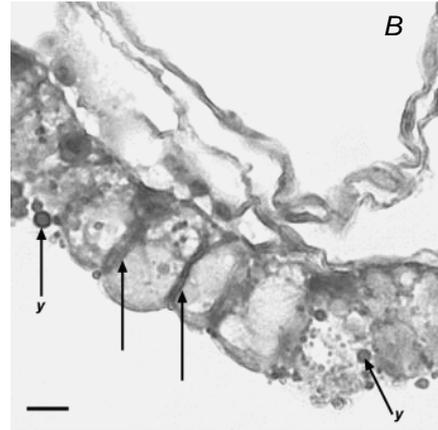
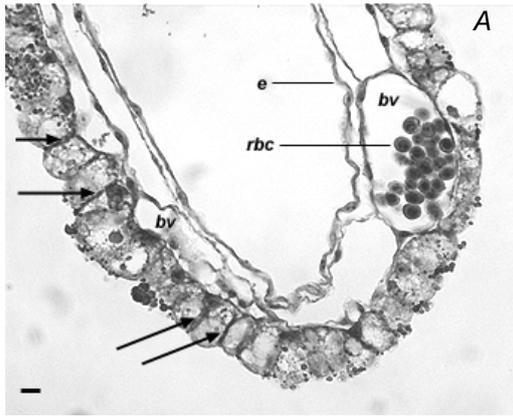
4 **A** – a representative transverse section through central part of the *area vasculosa* at 54h of
incubation showing sites of staining for carbonic anhydrase activity (arrows) at the intercellular
junctions of endodermal cells. *e* – epiblast; *bv* - blood vessel; *rbc* - erythrocytes.

6 **B** – detail from A showing sites of staining for carbonic anhydrase activity (arrows) at
intercellular junctions of endodermal cells and presumed yolk granules (*y*).

8 **C** – a representative transverse section through central part of the *area vasculosa* at 72h of
incubation showing some sites of staining for carbonic anhydrase activity (arrows) at
10 intercellular junctions of endodermal cells X, Y and Z which can be identified in D also. Arrow
heads identify additional sites of staining for carbonic anhydrase activity. Many endodermal
12 cells have a central vacuole (*v*); blood vessel – *bv*.

D – the adjacent section to C stained for carbonic anhydrase activity in the presence of 10^{-5} M
14 acetazolamide. Arrows identify the sites of staining for carbonic anhydrase activity identified in
C; *n* - the nucleus of an endodermal cell; *v* – vacuole; *bv* - blood vessel.

16 **E** – the detail from C showing the parallel-line nature of cobalt precipitation at the intercellular
junctions (arrows). The nucleus (*n*) of an endodermal cell (*e*) is visible and many cells have a
18 large central vacuole (*v*). Scale bars represent 10 μ m



2

Figure 4. The model proposed for the mechanism of sub-embryonic fluid production by the blastoderm of the Japanese quail between 54 h and 72 h of incubation. Stippled rectangles represent two endodermal cells enclosing a lateral intercellular space (*LIS*). Na^+/K^+ ATPase and carbonic anhydrase (CA) are located on the endodermal cell lateral plasma membranes, and show the proposed direction of ion movements: HCO_3^- into the intercellular space along with Na^+ , and K^+ and H^+ into the cytosol. A sodium hydrogen exchanger (NHE) is located on the plasma membrane facing the blood vessels, with H^+ moving out of the endodermal cell in exchange for Na^+ moving into the cytosol. A smaller proportion of the outwardly directed H^+ movement is due to V type H^+ ATPase located on this membrane. Inhibitors found to reduce fluid production – NEM, amiloride, ouabain and sulphonamides – are indicated in boxes with arrows pointing to the site of inhibition. Water is indicated as passing through the cytosol because of the large vacuoles observed in these endodermal cells.

2

