Effect of amphotericin B and gestational age on sodium transport across the rat visceral yolk sac placenta *in vitro*

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Summary. The transport properties of the rat visceral yolk sac placenta from Days 14·5 to 18·5 of gestation were studied *in vitro*. All tissues had a positive potential difference, fetal side relative to maternal side, and showed net Na transport towards the fetus. Basal short-circuit current and net Na flux increased rapidly with gestational age over the period studied. Amphotericin B applied to the maternal surface of the yolk sac stimulated current and net Na flux, indicating that the apical membrane Na permeability limited transport and revealing a reserve capacity for transport. Contrary to their basal values, current and Na flux following treatment with amphotericin were independent of gestational age.

Introduction

The rat visceral yolk sac provides an additional site for transfer between the fetal and maternal circulations, augmenting the usual mammalian (chorioallantoic) placenta (see Text-fig. 1). In the early stages of development, the rat embryo is dependent on the yolk sac, while in late gestation the placenta is pre-eminent. Thus the yolk sac is an important organ of exchange during organogenesis—as shown by the ability to culture embryos *in vitro* over this period under conditions in which nutrition cannot be supplemented by the placenta (New, 1978). In addition, there is a suggestion of a complementary relationship between the yolk sac and the placenta, with differential transport of dyes (Everett, 1935) and divalent cations (Wislocki, Deane & Dempsey, 1946).

Established functions of the yolk sac include the uptake of proteins and their subsequent selective transport or degradation (see Freeman, Beck & Lloyd, 1981) and transfer of immunoglobulins late in gestation (Brambell & Halliday, 1956). The yolk sac is important in the consideration of teratogenesis, either as a potential site of teratogen action or in terms of barrier function (Turbow, 1966; Beck, 1982). However, there is relatively little information available on other transport properties of this tissue, despite its importance to the developing embryo.

In the context of salt and water transport, the only previous substantive work in the literature is the study of Chan & Wong (1978) which established the late (17·5- to 21·5-day) yolk sac as an *in vitro* preparation. We have extended their findings recently (Ellory & Gibson, 1984) using 14·5-day tissues mounted in a specially designed Ussing chamber (Gibson, 1983). The present study represents an examination of Na transport in tissues from conceptuses of various gestational ages, and demonstrates the effect of development on salt transport.

Materials and Methods

Animals and dissection. Rats were kept under standard laboratory conditions. Females were placed with males overnight and fertilization was assumed if vaginal smears, taken on the following morning, contained spermatozoa (Day 0·5). When at the required stage of gestation, females were

killed by cervical dislocation, the abdomen opened and the gravid uterus removed. After removal from the uterus, the conceptuses were kept in aerated saline (see below) at 38°C until use. Yolk sacs were separated from the other fetal membranes, Reichert's and the amniotic membranes (Text-fig. 1), and, after dissection, pinned to the removable cassette of the Ussing chamber (for details see Gibson, 1983) which was then inserted between the two half-chambers. Half-chamber volume was about 0.8 ml and temperature was maintained at 38°C by circulating fluid through the water-jacket of the chamber. The area of exposed tissue could be varied between 0.3 and 0.8 cm².

Text-fig. 1. Schematic diagram showing the arrangement of fetal membranes in an early rat embryo (with part of Reichert's membrane removed (a), and detail of the visceral yolk sac epithelium, (b)).

The saline, Krebs–Ringer–bicarbonate, had the following composition, in mM: NaCl 118, KCl 4.7, MgSO₄ 1.17, CaCl₂ 2.56, NaHCO₃ 25, NaH₂PO₄ 1.13, D-glucose 10 and L-alanine 2, and was bubbled continuously with 95% O₂/5% CO₂ and adjusted with Tris to give a pH of 7.4 at 38°C.

Electrical measurements. All experiments were performed under short-circuit conditions using a conventional voltage-clamp. Transepithelial potential difference (p.d., mV) is expressed as the potential of the maternal side subtracted from the potential of the fetal side and was measured using calomel electrodes connected to the Ussing chamber via 3% agar–saline bridges ending about 2 mm from each side of the tissue. An external current was injected via a pair of Ag/AgCl electrodes and agar–saline bridges to short-circuit the p.d. to zero. Current is given in $\mu\text{A} \cdot \text{cm}^{-2}$, positive if flowing from the fetal to the maternal side. Resistance of the solution between the two voltage electrodes was compensated for automatically by the voltage-clamp apparatus. In practice, all tissues had a positive p.d. and a negative current.

Sodium flux measurements. Isotopic tracer fluxes of ²⁴Na and/or ²²Na were used to measure the unidirectional Na flux. All results for 14.5-day tissues were obtained using simultaneous bidirectional fluxes; ²⁴Na was used for maternal-to-fetal side fluxes (J_{MF}), and ²²Na for fetal-to-maternal side fluxes (J_{FM}) on the same tissue. At other gestational ages separate unidirectional ²²Na fluxes were sufficient to measure the net flux and were carried out on paired fetal tissues from the same mother and giving similar currents. To measure the bidirectional Na flux, ²⁴Na was used at 37–74 kBq · ml⁻¹ on the maternal side, with ²²Na at 3.7–7.4 kBq on the fetal side. Solutions were changed every 15 min using a double-barrelled syringe to obviate any pressure difference across the tissue. Stability of the preparation was improved by this latter precaution. ²⁴Na appearing in the fetal solution (J_{MF}) was counted immediately in a Packard Autogamma scintillation spectrometer.

and again after the decay of ^{24}Na to assess and then subtract the contribution of ^{22}Na to the total counts. Similarly, following ^{24}Na decay, ^{22}Na was counted after addition of 3 ml scintillant, Pico-FluorTM30, in a Packard Tricarb scintillation spectrometer. Unidirectional fluxes of ^{22}Na were counted, as above, using scintillant.

Both isotopes were obtained from Amersham International (Bucks, U.K.).

Amphotericin. Amphotericin was used as a saturated solution at $20\text{ }\mu\text{g. ml}^{-1}$ and was obtained as Fungizone (50 mg amphotericin, 41 mg sodium desoxycholate buffered with 20.2 mg sodium phosphate: E. R. Squibb & Sons, Inc., Princetown, NJ 08540, U.S.A.). Amphotericin is an ionophore acting on sterol-containing membranes to increase their permeability to small solutes, especially univalent ions (Lichtenstein & Leaf, 1965), including Na (e.g. Rose & Nahrwold, 1976). Maternal application of amphotericin causes intracellular Na of the yolk sac cells to reach electrochemical equilibrium with Na in the bathing medium and hence its concentration will rise markedly. Transport of Na across the basolateral membrane will be stimulated if apical membrane Na permeability, and thence intracellular Na concentration, was previously limiting. Under these conditions, the rate of Na pumping should be limited only by the density of Na pumps in the basolateral membrane or possibly by the ATP availability.

Statistics. Results are expressed as the mean $\pm$ s.e.m. of n observations. Comparisons are made by Student's t test.

Results

Initially, the data on electrical and flux studies on tissues from 14.5-day animals are presented in detail. Subsequently, tissues from different gestational ages are compared in terms of current and Na fluxes.

Tissues from 14.5-day conceptuses

The time course of current changes in 14.5-day tissues, mounted *in vitro*, is presented in Text-fig. 2. It is clear that 14.5-day tissues gave a stable current and p.d. over 1 h, as shown, but in other experiments, tissues continued to give stable values for at least 3 h. When ouabain (10^{-4} M) was applied to the fetal (or basolateral) side the current and p.d. fell rapidly to zero (Text-fig. 2), a result expected from the similar findings of Chan & Wong (1978) for the 19.5-day tissue. In conventional terminology for epithelia, therefore, the maternal side is apical and the fetal side is basolateral. Maternal (= apical) application of amphotericin ($20\text{ }\mu\text{g/ml}$) produced prompt stimulation of current, to values initially about 10-fold that of the control (or basal) values (Text-fig. 2b). The transient peak was followed by a decrease to a more stable level, about 5 times the control, by 30 min after addition of amphotericin. This current, like that in basal conditions, was ouabain-sensitive: similar stimulation of the current by amphotericin was not seen after prior treatment with ouabain to the fetal side. The transepithelial resistance, given by p.d./current, was about $350\text{ }\Omega\text{ cm}^2$ in basal conditions, tending to decrease after amphotericin to $250\text{ }\Omega\text{ cm}^2$ and increasing after ouabain to >1000 .

Bidirectional isotopic Na flux data are shown in Table 1. The control net Na flux was $0.46 \pm 0.05\text{ }\mu\text{mol. cm}^{-2}\text{. h}^{-1}$ towards the fetus, a value about 2–3 times greater than can be accounted for by the current, expressed in $\mu\text{equiv. cm}^{-2}\text{. h}^{-1}$. Na transport, therefore, must be accompanied by the net movement of some other ion. With ouabain treatment, net Na flux was only inhibited by about 50% after 90 min to $0.24 \pm 0.04\text{ }\mu\text{mol. cm}^2\text{. h}^{-1}$ ($n = 5$), despite the rapid effect on current (Text-fig. 2). Amphotericin caused a 4-fold increase in net Na absorption to $1.99 \pm 0.23\text{ }\mu\text{mol. cm}^{-2}\text{. h}^{-1}$ ($n = 5$).

Text-fig. 2. Effect of (a) ouabain applied to the fetal side, and (b) amphotericin applied to the maternal side of the 14.5-day rat yolk sac on the short-circuit current and potential difference. Values are mean $\pm$ s.e.m. for 5 observations.

Unidirectional flux data contained in Table 1 emphasize the large individual variation which necessitated the measurement of simultaneous bidirectional fluxes to reveal the small net Na flux at this gestational age. However, unidirectional fluxes do show that ouabain acted mainly by increasing J_{FM} , whilst amphotericin increased J_{MF} , presumably by stimulating the Na pump.

Table 1. Unidirectional Na fluxes in the yolk sac of the 14.5-day rat

Na flux ($\mu\text{mol} \cdot \text{cm}^{-2} \cdot \text{h}^{-1}$)				
	Control	Ouabain (10^{-4} M) to fetal side	Control	Amphotericin (20 $\mu\text{g}/\text{ml}$) to maternal side
J_{MF}	2.55 ± 0.68 (4)	2.65 ± 0.65 (4)	3.45 ± 0.83 (5)	4.96 ± 0.78 (5)
J_{FM}	2.10 ± 0.66 (4)	2.43 ± 0.67 (4)	3.08 ± 0.72 (5)	2.98 ± 0.59 (5)

Values are mean $\pm$ s.e.m. for the no. of observations in parentheses.

Effect of gestational age on electrical values and Na fluxes

The current as a function of gestational age (14.5, 15.5, 16.5, 17.5 and 18.5 days) is shown in Text-fig. 3. The results of Chan & Wong (1978) have been added for comparison and completeness. The trend shown by these authors, peak current at 19.5-days decreasing at higher and lower ages, continued in our study over earlier gestational ages. The apparent discrepancy at 17.5 days is not significant ($P > 0.1$) and probably reflects either variability or the rapid rate of change of current with age, necessitating measurement at the same time of day for accurate comparison. Text-figure 3 shows that, from 14.5 days to 18.5 days, the control or basal current could more than double over a 2-day period.

Amphotericin (20 $\mu\text{g/ml}$) was applied to the maternal side of tissues from Days 14.5 to 18.5 and the resulting currents (averaged over the subsequent hour) are shown in Table 2. At every gestational age, amphotericin stimulated current, implying that all tissues have a reserve Na pumping capacity not expressed in the basal situation. However, the size of this reserve capacity diminished with gestational age: at 14.5 days amphotericin stimulated current by 500%, whilst producing only a 35% stimulation at 18.5 days. Furthermore, the absolute current reached after amphotericin appeared to be the same from Days 14.5 to 18.5, despite the rising basal current.

The corresponding flux data are less complete (Table 2) but the trend shown is similar. For the three ages studied, flux after amphotericin was always about $2 \mu\text{mol} \cdot \text{cm}^{-2} \cdot \text{h}^{-1}$, whilst basal fluxes increased from 0.46 at 14.5 days to 1.20 at 16.5 days.

Text-fig. 3. Effect of gestational age on the short-circuit current of the rat yolk sac, values are means $\pm$ s.e.m. for 6–11 observations. The data of Chan & Wong (1978) are means $\pm$ s.e.m. for 4–6 observations.

Table 2. Effect of increasing gestational age on short-circuit current ($\mu\text{A} \cdot \text{cm}^{-2}$) and net Na fluxes ($\mu\text{mol} \cdot \text{cm}^{-2} \cdot \text{h}^{-1}$) under basal conditions and after maternal application of amphotericin

	Day of gestation				
	14.5	15.5	16.5	17.5	18.5
Current					
Control	3.8 ± 0.7 (5)	5.7 ± 0.7 (10)	10.0 ± 1.3 (7)	13.9 ± 1.1 (6)	17.0 ± 3.3 (8)
Amphotericin	24.4 ± 2.6 (5)	27.5 ± 1.1 (10)	22.7 ± 2.4 (7)	23.7 (1)	22.0 (2)
Net Na flux					
Control	0.46 0.05 (11)	0.07 ± 0.21 (4)	1.20 ± 0.35 (5)		
Amphotericin	1.99 ± 0.23 (5)	2.11 ± 0.34 (5)	2.15 ± 0.33 (2)		

Values are mean $\pm$ s.e.m. for the no. of observations in parentheses.

Discussion

The present paper seeks to establish the role of the rat yolk sac in salt transport during development, and to characterize any developmental changes occurring in its function during mid- and late-gestation.

We show that 14.5-day tissues behave in a qualitatively similar way to those of 19.5 days (Chan & Wong, 1978). In both cases, net Na flux towards the fetus exceeds the short-circuit current. Chan & Wong (1978) found that neither amiloride nor Cl replacement affected current, which suggests that the discrepancy does not result from Na/H exchange or NaCl co-transport. However, both these processes are non-electrogenic and it might be difficult to establish their presence by current measurements. Clearly, further studies on these and other possible transport processes are necessary. The smaller effect of fetal application of ouabain on net Na fluxes when compared to that on current is sometimes seen in other tissues (Ramos & Ellory, 1981) and could be due to a slow washout of Na from the tissue. The size of experimental error whilst measuring a very small flux will also contribute to an apparent ouabain-insensitive flux.

As gestation proceeds, basal net Na flux and current are seen to increase (Table 2; Text-fig. 3) but the possible regulatory mechanisms are unknown. The yolk sac could develop autonomously, with an intrinsic trigger affecting the basal properties, or an external signal, from either the mother or, more probably, the fetus, could be responsible. The culture of embryos *in vitro* (New, 1978) and the use of certain manipulations to produce selective death of the embryo whilst fetal membranes survive (e.g. culturing in high CO₂; Beck, 1983) provide obvious systems on which to test these possibilities.

The role of this net Na flux towards the fetus is not known. From analogies with intestine and kidney, tissues which develop from the same germ cell layer, possible functions are water and non-electrolyte transport (see Diamond & Bossert, 1967; Schultz & Curran, 1970). At present we know of no measurements of the Na dependence of water, glucose or amino acid fluxes. One can estimate the total volume that the rat yolk sac is able to transport (Table 3) assuming isotonic flow accompanying the Na flux, and compare this value with the increasing fluid volume in the conceptus (using data from Tam & Chan, 1977). Na transport by the yolk sac is easily able to accommodate this volume. The apparent excess Na movement can easily be accounted for by hypertonic water flow or the need to replace water lost by some other mechanism, e.g. by a hydrostatic pressure difference across the yolk sac tending to keep it distended; a deficit would be harder to explain. However, a more specific role for Na is likely and, in this context, measurements of non-electrolyte fluxes in the presence and absence of Na would be of value.

Finally, the significance of stimulation of net Na flux and current by amphotericin should be considered. Amphotericin, in this case, has the principal effect of increasing apical membrane Na

Table 3. An estimate of water transported by the rat yolk sac

	14.5–15.5 days	15.5–16.5 days	16.5–17.5 days
Change in conceptus volume* (ml/day)	0.244	0.393	0.478
Estimated yolk sac area† (cm ²)	5.8	7.4	10.6
Na flux towards fetus‡ (μmol/day)	81	169	305
Estimated volume transported§ (ml/day)	0.54	1.12	2.03

* Maximum change in conceptus volume between the 2 days, using data from Tam & Chan (1977), = (Δ wet wt of fetus – dry wt) + (Δ wet wt of placenta and membranes) + (Δ wt, extraembryonic fluid).

† Estimated as the area of a sphere $2r = 1.3$ at 14.5 days, 1.4 at 15.5 days, 1.65 at 16.5 days and 2.0 at 17.5 days, in cm. Area is averaged over the 2-day period.

‡ Na flux towards fetus = average Na flux over the 2 days (μmol · cm⁻² · h⁻¹) from basal values, in Table 2, × 24 × yolk-sac area.

§ Volume transported, assuming Na concentration is 150 mM (Na flux/150 in ml).

permeability (Rose & Nahrwold, 1976) and thence raising the intracellular Na concentration. Stimulation of current and net Na flux in these conditions indicates that the apical membrane Na permeability was previously limiting to Na pumping, and reveals a large reserve capacity for Na transport not expressed in basal conditions. The transient current could be caused by diffusion potentials being established across the intercellular junctions following the increased rate of Na pumping at the basolateral cell membrane. A contribution from a decreasing Na pump rate during this period is also possible. The extent of the reserve capacity for Na transport decreases with increasing gestational age. These results suggest the presence of a possible second regulatory mechanism, concomitant with the developmental changes occurring through gestation (see above). It would allow for the presence of hormonal control signals from the mother or, more probably, the fetus to adjust the yolk sac properties to that required, via action on the apical membrane permeability. One can envisage the advantage of such regulation acting as a fine control, perhaps when superimposed upon an automatic change in property of the yolk sac with gestational age. Further, it seems, from Table 2, that the maximum rate of Na transport by this tissue is independent of gestational age, although the basal flux increases with age. The results could be interpreted in terms of a fixed basolateral Na pumping capacity. They suggest that, during the developmental period studied in this paper, there is no change in Na pump density within the yolk sac tissue, whilst Na transport shows a considerable reserve, probably modulated at the level of apical Na permeability.

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