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PROPERTIES OF SURFACE AND JUNCTIONAL MEMBRANES OF EMBRYONIC CELLS ISOLATED FROM BLASTULA STAGES OF *XENOPUS LAEVIS*

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SUMMARY

1. Some membrane properties of endoderm and mesoderm cells isolated from late blastula stages of *Xenopus laevis* have been examined using electrophysiological techniques.

2. Cells were isolated by treatment of whole embryos with Ca-free EDTA containing media, or mechanically by micro-dissection, and cultured in Ca-containing Holtfreter solution (60 mM-NaCl) or Ringer solution (120 mM-NaCl).

3. Membrane potentials lay between -6 and -84 mV; specific membrane resistances ranged from 500 to 29,000 Ω cm^2; there was no difference between EDTA isolated and mechanically isolated cells.

4. Relative and absolute cation and anion conductances varied from cell to cell. Some cells were anion impermeable; the cation conductance ranged from 35 to 300 μmho/cm^2.

5. The resting potential of some cells was largely determined by the concentration gradient and membrane permeability of K ions. In other cells the potential was maintained either by some other ion or by an electrogenic pump. $[K]_i$ came to $\sim$ 130 mM in Ringer solution (the value pertaining in the intact embryo) and $\sim$ 60 mM in Holtfreter solution.

6. In most pairs and small clumps of cells ionic current spread from one cell to the next; some single cells and groups of cells were uncoupled from their neighbours.

7. The junctional resistance lay between 10^5 and 10^8 Ω; it behaved as a linear resistor in most cell pairs studied. In three pairs the intercellular junction showed rectifying properties.

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8. By the late blastula stage of development presumptive endoderm and mesoderm cells form a heterogeneous population with widely varying passive membrane properties. The significance of these findings is discussed in relation to current hypotheses for the formation of spatial patterns during differentiation.

INTRODUCTION

At early stages of embryonic development all cells are in ionic communication with each other (squid embryo: Potter, Furshpan & Lennox, 1966; amphibian: Ito & Hori, 1966; Palmer & Slack, 1970; Warner, 1970; fish: Bennett & Trinkaus, 1970; chick: Sheridan, 1966; starfish: Tupper & Saunders, 1972; tunicate: Miyazaki, Takahashi, Tsuda & Yoshi, 1974); electrotonic current spread between cells of different eventual fates persists after morphological differentiation has begun (chick: Sheridan, 1968; amphibian: Warner, 1973). Grafting experiments have shown that cells of the early embryo mutually interact and influence each other's fate (e.g. amphibian: Spemann, 1938; Tarin, 1971; Cooke, 1972) and the low resistance intercellular pathway has been suggested as the channel for these interactions.

The membrane potential of surface cells of the amphibian embryo are closely similar (Slack & Wolpert, 1972) and the question arises whether this uniformity is the consequence of short circuiting of the properties of individual cells along the intercellular pathway or reflects a cell population with homogeneous membrane properties. Nieuwkoop (1969*a*, *b*) has suggested that by the blastula stage the cells already form a population with diverse developmental fates. We have therefore examined whether cells of the endoderm and mesoderm, isolated from blastula stage *Xenopus laevis* embryos (Curtis, 1957; Jones & Elsdale, 1963), display similar membrane properties when separated from their neighbours.

Information on the ionic permeability of the barrier between each embryonic cell might show how the intercellular junction plays a part in controlling cellular interactions early in development. Some measurements of input and transfer resistance in pairs of cells, allowing calculation of the relative magnitude of surface and junctional membrane resistances, are therefore also included in this paper.

A preliminary report of some of this work has appeared (Slack & Warner, 1969).

METHODS

Embryos were obtained by injection of chorionic gonadotrophin into mature adults of *Xenopus laevis*. The embryos were staged according to the Normal table of *Xenopus laevis* (Nieuwkoop & Faber, 1956) or by reference to Developmental Tables given in Rugh (1961*a*).

Jelly and vitelline membranes were stripped from embryos at the late blastula stage (stages 8 and 9, Nieuwkoop & Faber, 1956) using fine watchmaker's forceps. Disaggregation was achieved by soaking in Ca-free Holtfreter solution (60 mM-NaCl; 1 mM-KCl; 1 mM-Tris-hydroxymethylamino-methane (Tris)buffer) or Ringer solution (120 mM-NaCl; 2·5 mM-KCl; 2 mM Tris) containing 1 mM EDTA (ethylenediamine tetra-acetic acid) at pH 8·0, for the minimum length of time necessary to produce a number of single cells. This varied greatly from one embryo to the next and ranged from 10 to 45 min. Unpigmented cells of the endoderm and mesoderm were returned to Ca-containing Holtfreter solution (1 mM-$CaCl_2$) or Ringer solution (2 mM-$CaCl_2$) at pH 7·4 and measurements begun at noted times after removal from EDTA-containing solution. Alternatively loosely attached cells from the inner surface of the vegetal pole, which separated without recourse to chemical treatment, were used. Cells isolated in this way are referred to as 'mechanically isolated' in the text.

The cells were transferred to a black millipore filter on the bottom of a small bath (volume 2 ml.) through which solutions flowed at between 3 and 12 ml./min. They adhered firmly to the millipore filter, probably by inserting pseudopodia into the filter holes (Wartiovaara, Lehtonen, Nordling & Saxen, 1972), but would not attach to glass or plastic surfaces. The composition of the solution flowing through the bath was varied using a multiple tap of the kind described by Hodgkin & Horowicz (1960); alterations in ionic composition of the bathing medium are detailed where appropriate in the text. The survival of the isolated cells was improved by using sterile solutions. The temperature of the superfusing solution was maintained at 21–23° C.

Membrane potentials and resistances were recorded using fine tipped microelectrodes (30–100 MΩ resistance), filled with 0·8 M-K citrate with tip potentials < -8 mV in Holtfreter solution and < -5 mV in Ringer solution. The Ag^+–AgCl indifferent electrode made contact with the bath via an Agar–Holtfreter or Agar–Ringer half cell. Potentials were recorded using conventional electrophysiological techniques and displayed on an oscilloscope and pen recorder.

Cell diameters were measured through the microscope eyepiece of a Zeiss stereo IV dissecting microscope using a calibrated eyepiece graticule.

RESULTS

Previous work on cells isolated from amphibian embryos has been done in a medium containing 60–80 mM-Na (Holtfreter, 1943; Curtis, 1957; Barth & Barth, 1959, 1963; Jones & Elsdale, 1963), although the ionic composition of the intercellular fluid in *Xenopus* more closely resembles Ringer solution (Slack, Warner & Warren, 1973). The measurements reported in this paper were therefore made in Holtfreter (60 mM-Na^+) and Ringer solutions, to allow comparison between previous experimental conditions and those more likely to pertain in the intact embryo. All measurements were made on unpigmented cells from the endoderm or mesoderm.

Measurements in Holtfreter solution

Recovery from disaggregation procedures. Removal of external Ca ions from the bathing medium produced a fall in membrane resistance and membrane potential (Palmer & Slack, 1970); the embryo then disaggregated into single cells. Fig. 1 shows membrane potentials recorded

in vegetal pole cells, isolated in this way from a single embryo at stage 8, plotted at different times after return to Ca-containing Holtfreter solution at pH 7·4. During the first hour 60 % of the cells had resting potentials below − 20 mV. After 1½ hr all cells sampled had potentials of − 20 mV or more; there was no further change in membrane potential distribution. Measurements on EDTA isolated cells were therefore delayed until 1½–2 hr after return to Ca^{2+}-containing media. Mechanically isolated cells showed no alteration in properties with time after disaggregation (up to 4 hr) and responded similarly to EDTA isolated cells when challenged with disaggregating medium.

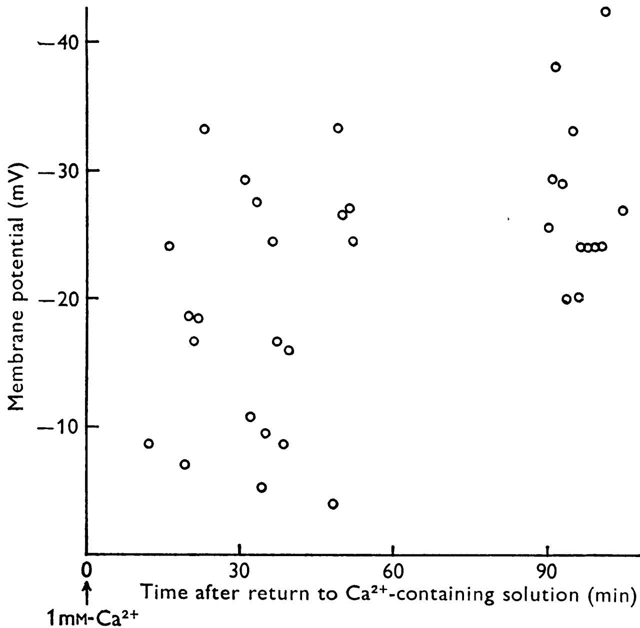

Fig. 1. Membrane potentials in cells isolated from late blastula embryo at different times after removal from Ca^{2+}-free Holtfreter solution containing 1 mM-EDTA. Ordinate: membrane potential (mV). Abscissa: time after return to Ca^{2+}-containing Holtfreter solution.

The cells cyclosed and divided during recording regardless of method of disaggregation or ionic strength of the culture medium. Cells kept in long term culture attached, flattened and differentiated, suggesting that the disaggregation procedure did not produce permanent damage (P. A. Jackson, A. Messenger & A. E. Warner, unpublished).

Membrane potentials and membrane resistances. The cells were fragile, often swelling and bursting after micro-electrode insertion. Nevertheless in many cases the recorded membrane potential remained steady (Fig. 2*a*).

Frequently there was a small, gradual increase in membrane potential (Fig. 2*b*), but on occasion the membrane potential suddenly increased sharply after it had apparently become steady. In the example illustrated in Fig. 2*c* the initial membrane potential was −16 mV; after a delay, the potential rose spontaneously settling close to −50 mV. Cells could have either high (up to −65 mV) or low (between −15 and −20 mV) initial membrane potentials, but spontaneous, large increases in potential were only seen in cells whose initial potential lay below −25 mV; subsequent treatment with ouabain (10^{-4} M) did not reverse the potential change. The reason for this behaviour is obscure, but it could reflect some change in cell membrane properties consequent upon the introduction of a non-specific ionic leak on micro-electrode insertion. It occurred in both EDTA isolated and mechanically isolated cells.

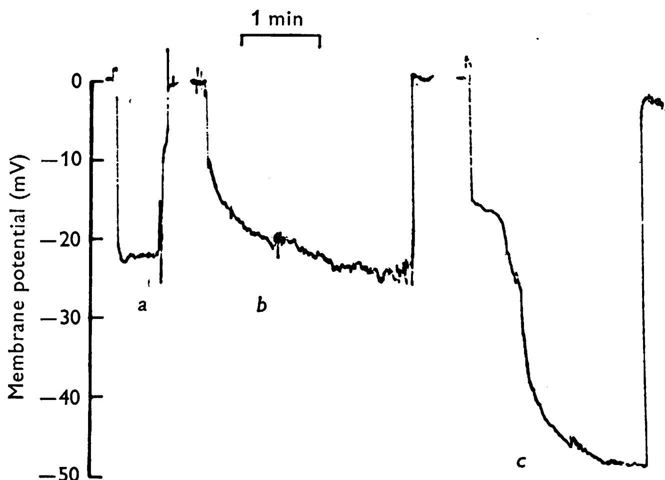

Fig. 2. Pen records of membrane potentials recorded in three cells isolated and maintained in Holtfreter solution. Ordinate: membrane potential (mV).

Fig. 3 plots frequency histograms of maximum membrane potentials recorded from mechanically isolated cells (*A*) and EDTA isolated cells (*B*). In both cases the values are widely spread, ranging from −5 to −65 mV. The proportion of cells with resting membrane potentials below −25 mV was greater in those cells isolated with EDTA. This difference might arise because some of these cells do not fully recover from treatment with a Ca chelator, or are liable to damage during micro-electrode insertion. To assess the contribution of damage the recorded membrane potential was plotted against cell diameter for each population of Fig. 3 on the assumption that the effect of a non-specific depolarizing leakage current would

be greatest in small diameter cells. Such plots showed no correlation (diameter range 40–275 μm).

Input resistances were determined by injecting a small hyperpolarizing current pulse one second long, through a second micro-electrode and measuring the height of the resultant electrotonic potential. For conversion of input resistance to specific membrane resistance each cell was treated

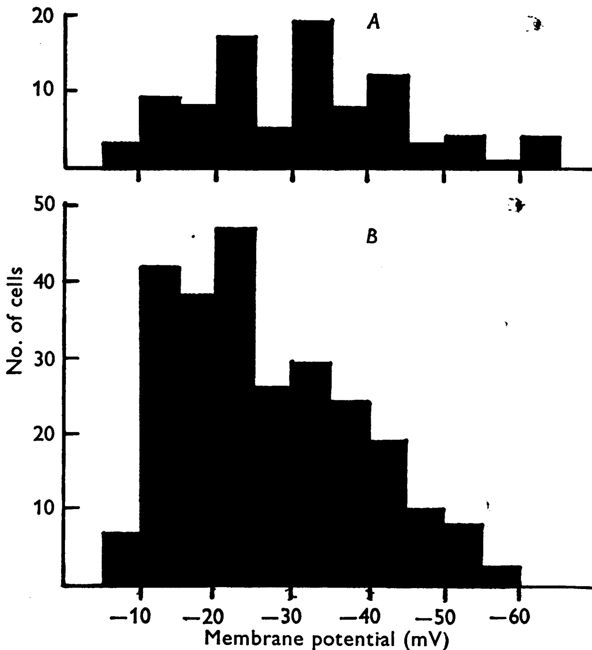

Fig. 3. Frequency histograms of membrane potentials recorded from mechanically isolated cells (*A*) and EDTA isolated cells (*B*). Ordinates: no. of cells in each class. Abscissae: membrane potential (mV). Cells in Holtfreter solution.

as a sphere. Frequency plots of specific membrane resistance for mechanically isolated and EDTA isolated cells are given in Fig. 4*A* and *B*. Membrane resistances varied widely, ranging from 560 (similar to the squid axon; Hodgkin, 1951) to 29,000 Ω cm^2 (similar to smooth muscle; Tomita, 1967). The range was the same regardless of method of disaggregation, with most cells lying between 1000 and 10,000 Ω cm^2. If the higher proportion of EDTA isolated cells with low membrane potentials (Fig. 3*B*) reflected a larger number of damaged cells, then this might be revealed as a low specific membrane resistance in those cells with low membrane potentials. Fig. 5 plots specific membrane resistance against membrane

potentials for the EDTA isolated cells. Only two cells in the population have membrane resistances below 1000 Ω cm²; one had a membrane potential of −5 mV, the other −28 mV. A similar range of membrane

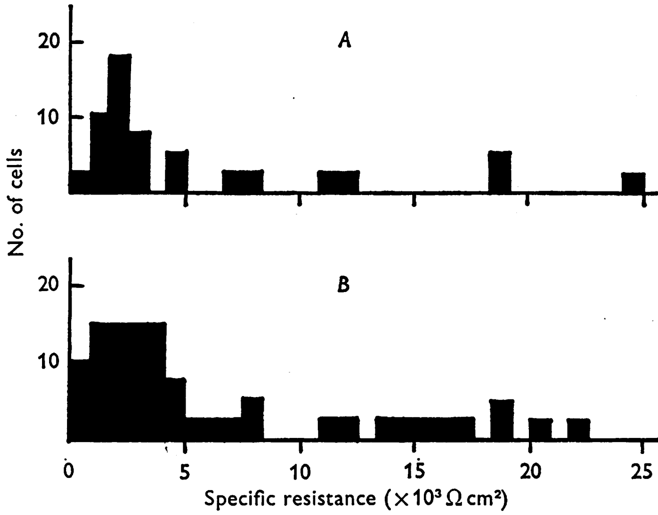

Fig. 4. Frequency histograms of specific membrane resistances measured in mechanically isolated cells (*A*) and EDTA isolated cells (*B*). Ordinates: no. of cells in each class. Abscissae: specific membrane resistance (Ω cm²).

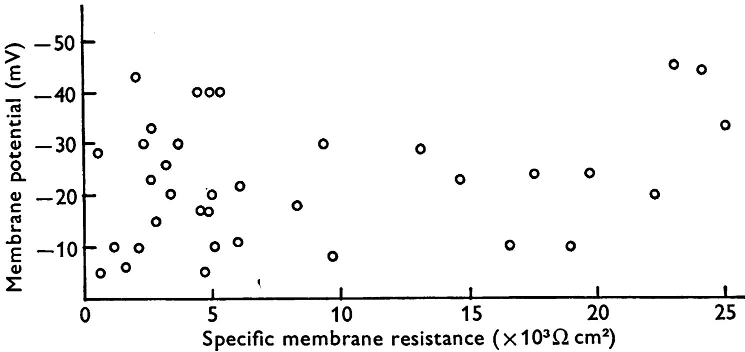

Fig. 5. Plot of membrane potential (ordinate) against specific membrane resistance (abscissa) for the population of EDTA isolated cells illustrated in Fig. 3*B*. Note absence of correlation.

potentials pertains at all other membrane resistances. The absence of a direct relationship between membrane potential and specific membrane resistance suggests that a low membrane potential does not necessarily indicate cell damage, but may result from differences in intracellular

concentrations or relative permeabilities of the potential determining ions. The difference between the two populations could, therefore, be the consequence of difference in sample size only (EDTA isolated $n = 251$; mechanically isolated $n = 93$) (cf. p. 109).

Membrane capacities, measured from the time constant of the membrane (the time taken for the electrotonic potential to reach 67% of its final value) ranged between 1 and 3 μF/cm^2.

The membrane voltage–current relation. Fig. 6 shows examples of voltage–current relations recorded in mechanically and EDTA isolated cells. The cell illustrated in *A* (mechanically isolated) had an initial resting potential of -34 mV and a resting membrane resistance of 9200 Ω cm^2. The final voltage displacement from the resting potential produced by a 1 sec current pulse is plotted against the injected current. As the internal potential was made less negative the membrane conductance rose. Rectification in this direction might be expected on the basis of the constant field equation (Goldman, 1943; Hodgkin & Katz, 1949), but the goodness of the fit cannot be assessed since the intracellular concentrations and relative ionic permeabilities of the current carrying ions are not known for this cell. Fig. 6*B* shows the voltage–current relation recorded in an EDTA isolated cell. The initial resting potential was -40 mV and resting membrane resistance 4200 Ω cm^2. The resistance changed little throughout the potential range ($+60$ to -100 mV). Other cells showed either linear or outwardly rectifying voltage–current relations. There was no dependence on the method of cell isolation.

The membrane resistance recorded for large depolarizing and hyperpolarizing pulses ($> \pm 100$ mV) was not always stable and time dependent variations in membrane conductance were sometimes observed, but no systematic pattern emerged.

The K sensitivity of the membrane potential. The relative permeability of the membrane to K ions can be assessed by measuring the membrane potential at different extracellular concentrations of K (see Adrian, 1958; Hodgkin & Horowicz, 1960). Alterations in intracellular K concentration consequent upon variations in K in the extracellular solution were avoided by replacing NaCl in Holtfreter solution with equimolar amounts of KCl. If the cell under study is anion impermeable then no net movements of K will occur. For an anion permeable cell there will be net entry of KCl, accompanied by water, but if K^+ ions make up approximately one half the osmotically active intracellular particles, which seems likely on the basis of ion and water distribution between cells and intercellular spaces in the whole embryo (Slack *et al.* 1973), the internal K concentration should not alter significantly (see Boyle & Conway, 1941).

The wide spread of resting membrane potentials made sampling of the potential of a number of cells at each K concentration uninformative. The intracellular potential was therefore recorded continuously while the K concentration in the superfusing fluid was raised stepwise from 0·7 to 50 mM. The membrane potential was read 15 min after each solution

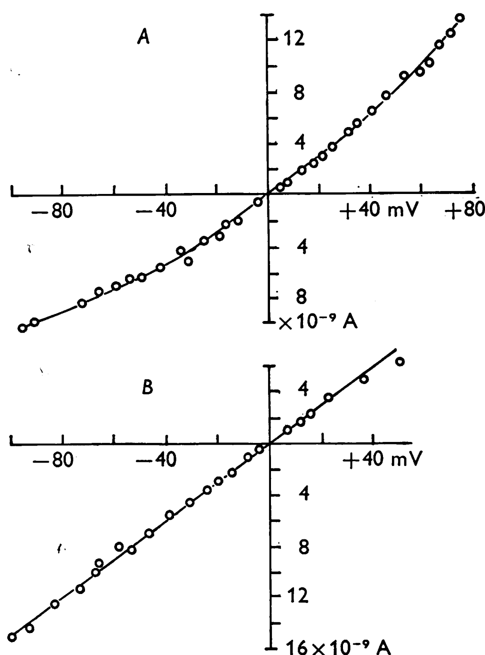

Fig. 6. Voltage–current relations measured in single cells isolated in Holtfreter solution. *A*, mechanically isolated cell, resting potential −34 mV. *B*, EDTA isolated cell, resting potential −40 mV. Ordinates: injected current (*A*). Abscissae: deflexion from resting potential (mV).

change, when the potential had become steady. Measurements in at least four external K concentrations were necessary to define the relation between internal potential and $[K]_0$ for each cell. On eight occasions the potential change on return from 50 to 0·7 mM $[K]_0$ was also monitored; the membrane potential came back to within 5 mV of the initial potential. The minimum duration of a satisfactory run was therefore about 1 hr; consequently many partial runs, but few complete runs were obtained. Two of these are illustrated in Fig. 7. The ordinates give the recorded membrane potential, the abscissae the concentration of K in the superfusing fluid on a logarithmic scale. In *A* the initial resting potential was −36 mV. The potential began to fall when the K concentration was raised to

5 mM, reaching −5 mV at 50 mM external K. Above 10 mM external K the potential dropped 40 mV for a tenfold increase in external K concentration. Five other cells with initial resting potentials between −24 and −60 mV behaved similarly.

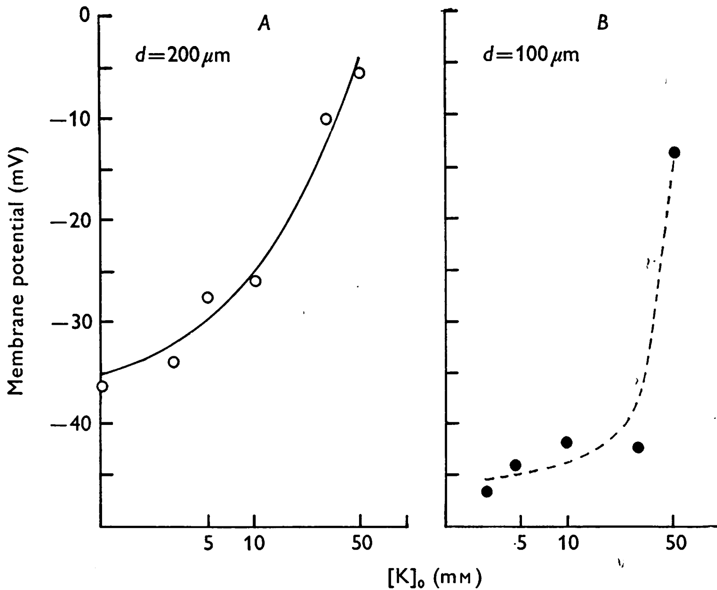

Fig. 7. Sensitivity of the membrane potential to variations in extracellular K concentration in cells isolated and maintained in Holtfreter solution. Both cells mechanically isolated. Ordinates: membrane potential (mV). Abscissae: extracellular concentration of K (mM). Na in Holtfreter solution substituted mole for mole by K. Continuous line in *A* drawn according to Goldman equation:

$$E_m = \frac{RT}{F} \ln \frac{[K_o] + \alpha[Na]_o}{[K]_i + \alpha[Na]_i} \quad \text{for} \quad [K]_i = 60 \text{ mM}; [Na]_i = 6 \text{ mM and } \alpha = (P_{cat}/P_K)$$

= 0·24. Dashed line in *B* drawn through points by eye.

The maximum potential change produced by a tenfold increase in $[K]_0$ lay between 32 and 47 mV, suggesting that the membrane permeability to ions other than K (P_{cat}) was sufficiently great to influence the level of the resting membrane potential. If anions are passively distributed, and contributions from active transport processes can be neglected, estimates of the relative permeability ratio P_{cat}/P_K can be made from linear regression plots of $[K]_0$ against exp (VF/RT), provided the intracellular concentrations of the potential determining ions do not change during the

experiment. P_{cat}/P_K is calculated from the ratio of the slope of such plots to the intercept. P_{cat}/P_K, determined in this way for the cell of Fig. 7*A*, came to 0·24; other cells gave values ranging from 0·11 ($E_m = -60$ mV) to 0·25 ($E_m = -28$ mV).

The membrane potential should approach zero when ($[K]_o + P_{cat}/P_K$ $[cat]_o$) is equal to ($[K]_i + P_{cat}/P_K$ $[cat]_i$); the graph plotted in Fig. 7*A* sets ($[K]_i + P_{cat}/P_K$ $[cat]_i$) close to 60 mM. Analysis of K and Na in whole embryos at the late blastula stage showed the intracellular K concentration to be approximately 110 m-equiv K^+/l. cell H_2O and Na activity to be 13 m-equiv Na^+/l. cell H_2O (Slack *et al.* 1973). Cells isolated and maintained in Holtfreter solution (60 mM-Na^+, 1 mM-K^+) either have lost 50 % of their intracellular K, or have had their intracellular contents diluted because water enters when the cells are transferred from the intercellular fluid (Na ~ 105 mM; Slack *et al.* 1973) to Holtfreter solution. If it is assumed that the intracellular K concentration and Na activity have fallen to one half because of water entry, then the results of Fig. 7*A* can be fitted by the Goldman equation (Goldman, 1943). The line drawn through the points has been calculated with $[K]_i$ set at 60 mM, a^i_{Na} set at 6 mM and P_{cat}/P_K at the value determined above. In all cells for which a value for P_{cat}/P_K could be calculated the results were similarly fitted by the Goldman equation; values for $[K]_i$ lay between 50 and 80 mM.

In a proportion of cells (five out of twenty-five) the membrane potential was surprisingly insensitive to variations in extracellular K. Fig. 7*B* illustrates a complete run carried out on such a cell. The resting potential, measured in 3 mM-K, was -46 mV. At 30 mM external K the potential was still greater than -40 mV; raising the external K still higher to 50 mM brought the potential down to -13 mV. No ready explanation can be found for this relative insensitivity to increases in $[K]_o$. Some ion other than K may here be responsible for maintaining the resting potential, possibly Cl ions. Alternatively the potential could be maintained in the face of a falling gradient for K by the activity of an electrogenic pump. These possibilities could not be tested by repeating the measurements of membrane potential at different $[K]_o$ for the insensitive cells in the presence of a nominally 'impermeant' anion or in the presence of ouabain, since they could only be identified physiologically, and micro-electrode insertion could not be held for sufficiently long after identification.

On occasion Na in the extracellular solution was replaced by choline to check whether this influenced the relation between $[K]_o$ and E_m. This treatment rendered the cell surface rigid, making insertion of a micro-electrode without infliction of severe damage impossible. Removal of external Na might induce contracture in the contractile cytoplasm which lies beneath the plasma membrane (Baker & Warner, 1972; Gingell, 1970; Selman & Perry, 1970; Schroeder, 1970), as occurs in strips of muscle taken from frog heart ventricle (Luttgau & Niedergerke, 1958).

Micro-electrode insertion was also found to be difficult in the high K-containing solutions; in 50 mM-$[K]_o$ the cells divided noticeably more frequently. In order to do these experiments it was therefore necessary to insert the micro-electrode in Na-containing solution and then change to one in which choline replaced Na. There was no obvious difference in the pattern of response to changes in $[K]_o$ under such conditions.

The relative membrane conductance to cations and anions. The measurements described so far suggest that at the blastula stage isolated embryonic amphibian cells do not form a homogeneous population with respect to their passive electrical properties. We therefore tested whether differences in relative permeability to cations and anions might be responsible for some of the heterogeneity.

TABLE 1. Membrane conductance of single cells isolated and maintained in Holtfreter solution in the presence and absence of Cl ions

Cell no.	g_m (μmho/cm^2)	g_{cat} (μmho/cm^2)	g_{Cl} (μmho/cm^2)	g_{cat}/g_{Cl}
i	61·5	46	15	3·0
ii	110	35	75	0·46
iii	326	250	76	3·3
iv	215	115	100	1·15
v	37	37	0	—
vi	300	300	0	—
vii	45	45	0	—
Mean	156·4	118	66·6	—
± S.E. of mean	± 46·4	± 43	± 13·6	—

The change in membrane conductance produced when extracellular Cl ions are replaced by a relatively impermeant anion has been used to estimate the relative proportions of membrane current carried by K and Cl ions. In the absence of a convenient way of estimating the relative permeability of the embryonic cells to different anions, the methylsulphate anion, which proved a satisfactory relatively impermeant substitute for Cl ions in frog muscle (Hutter & Noble, 1960; Hutter & Warner, 1967) and the slightly larger ethylsulphate anion, have been used.

In a number of cells replacement of extracellular Cl in Holtfreter solution by methyl or ethylsulphate produced a prompt, transient fall in membrane potential accompanied by a rise in membrane resistance. Return to Cl containing media restored the initial high conductance. Some cells showed no alteration in either membrane potential or membrane resistance during the change in extracellular anion. Provided the methylsulphate anion does not carry current across the cell membrane these results show that some cells in the population are not permeable to Cl ions.

Table 1 details complete measurements made in seven of the cells. The Cl conductance (g_{Cl}) was calculated by assuming the membrane conductance (g_m) to be represented by two independent, parallel channels, g_{cation} and g_{anion}. Not only does the total membrane conductance vary widely from one cell to the next (range 37–326 μmho/cm^2) but also the absolute values of the individual cation and anion conductances. Three cells showed no detectable Cl conductance. Since the resting membrane conductances in these three cells varied by a factor of ten, differences in relative anion and cation permeabilities cannot form the sole basis of the cellular heterogeneity.

Measurements in Ringer solution

Some measurements were repeated on cells disaggregated and subsequently superfused with Ringer solution. The osmolarity of Ringer solution is close to that of plasma and cells disaggregated in Ringer rather than Holtfreter (~ half-strength Ringer) solution suffered less lysis during disaggregation in EDTA-containing solution, were less fragile and recovered a high membrane potential on return to Ca-containing solution in a few minutes. In Ringer solution the cells may swell less during the non-specific permeability increase produced by EDTA treatment.

Membrane potentials and resistances. Fig. 8 plots the frequency distribution of membrane potentials recorded in Ringer solution. EDTA isolated and mechanically isolated cells have been plotted as one population.

The measurements in Holtfreter solution suggested the difference in membrane potential distribution between EDTA isolated and mechanically isolated cells to be the consequence of sample size. This conclusion was reinforced by the finding that in Ringer solution the greater number of cells with potentials less than -25 mV fell in the mechanically isolated rather than the EDTA isolated population.

A higher proportion of cells than in Holtfreter solution had potentials between -35 and -40 mV, and a number of cells had potentials above -65 mV (cf. Fig. 3). The most likely explanation for this difference is the lower fragility of cells in Ringer solution.

As in Holtfreter solution the specific membrane resistances ranged between 800 and 29,000 Ω cm^2. A greater membrane conductance might be expected in Ringer solution if the membrane permeabilities do not change. In a few experiments the membrane conductance of the same cell was measured consecutively in each of the two solutions showing the membrane conductance to be greater by 30% in Ringer solution. The range of cell membrane resistances was sufficiently wide to mask any systematic difference between the two populations.

The K sensitivity of the membrane potential. Fig. 9 illustrates relations between membrane potential and extracellular K concentration

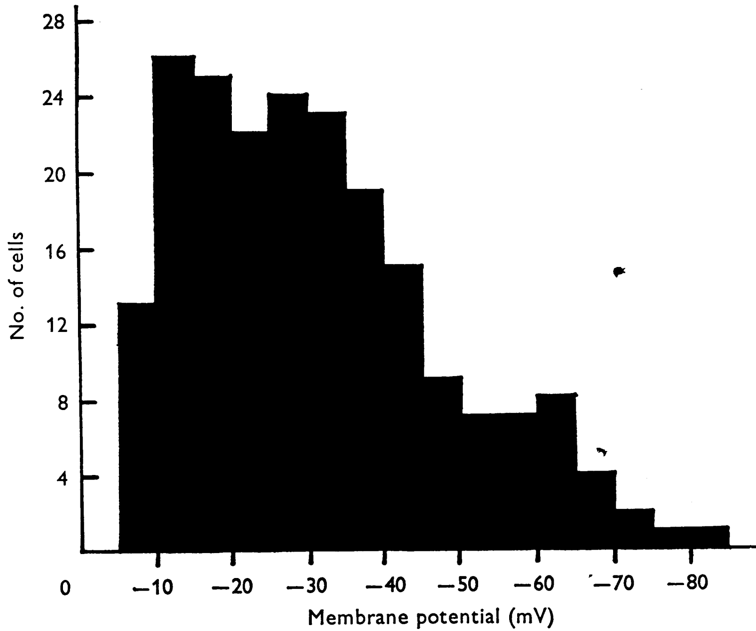

Fig. 8. Frequency histogram of membrane potentials recorded from cells isolated and maintained in Ringer solution. Ordinate: no. of cells in each class. Abscissa: membrane potential (mV). Mechanically isolated cells and EDTA isolated cells plotted as one population.

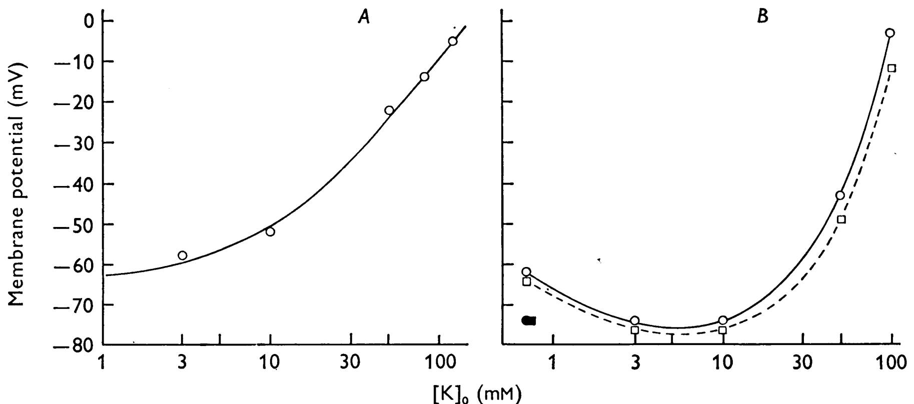

Fig. 9. Effect of increasing extracellular K concentration on membrane potential of a single cell (*A*) and a pair of cells (*B*) isolated and maintained in Ringer solution. Ordinates: membrane potential (mV). Abscissae: extracellular K concentration (mM). Continuous line in *A* drawn according to Goldman equation for $[K]_i = 145$ mM; $[Na]_i = 13$ mM; $\alpha = 0{\cdot}09$. Lines in *B* drawn through points according to eye. Cell pair illustrated in *B* electrotonically coupled: coupling ratio = 0·84. Filled circles give potential on return to low $[K]_o$.

obtained in three cells isolated in, and subsequently superfused with, Ringer solution. *A* shows measurements taken from a cell with a resting potential of -58 mV at 3 mM-$[K]_o$. The membrane potential fell as K^+ was substituted for Na^+, reaching -5 mV at 120 mM-$[K]_o$. Between 30 and 120 mM-$[K]_o$ the potential fell 50 mV for a tenfold increase in extracellular K concentration. By extrapolation the zero membrane potential value would have occurred at about 150 mM-$[K]_o$. On return to low $[K]_o$ (1 mM) the initial, high resting potential was restored. Estimates of the relative permeability ratio P_{cat}/P_K were made from linear regression plots of $[K]_o$ against exp (VF/RT). The results illustrated in Fig. 9*A* gave a value of 0·09 to P_{cat}/P_K and eight other cells with resting potentials between -65 and -28 mV behaving similarly gave values ranging from 0·08 to 0·2.

As in Holtfreter solution, some cells were relatively insensitive to increases in the extracellular concentration of K; in one cell an increase of $[K]_o$ from 0·7 to 30 mM and then to 100 mM failed to shift the potential from its initial value of -45 mV. Fig. 9*B* shows results from a pair of cells in which the increase in $[K]_o$ initially produced a rise, rather than a fall in membrane potential. The two cells were electrotonically coupled to each other (Table 2, pair *B*7) and had resting potentials in 0·7 mM-$[K]_o$ of -62 and -64 mV. At 3 and 10 mM-$[K]_o$ the potential of both cells rose, and then fell together at higher K concentrations. A difference in membrane potential between the two cells was maintained over the whole concentration range, rising to 10 mV at 100 mM-K. This suggests that the presence of a low resistance intercellular junction between two cells does not necessarily lead to equivalence of membrane potential, but allows each cell to respond according to its own specific properties. The solid points in Fig. 9*B* give the membrane potential measured on return to 0·7 mM-$[K]_o$.

It is again necessary to fall back on some potential determining ion other than K, or a contribution to the membrane potential from an electrogenic pump to explain the maintenance of a high membrane potential in the face of a falling K gradient. The finding that in some cells, e.g. Fig. 9*B*, the membrane potential rose for a moderate increase in extracellular K would support this notion, since it is known that the Na–K exchange pump, which is stimulated by an increase in extracellular K, can make an electrogenic contribution to the membrane potential, particularly in cells with a high resting membrane resistance (Thomas, 1972). On return to a low extracellular $[K]_o$ the membrane potential of all the cells studied returned to a value close to or greater than the initial resting potential; the cells of Fig. 9*B* returned to a potential 10 mV greater than the initial value in 0·7 mM-$[K]_o$. Either the intracellular K concentration has risen, or the electrogenic component of the membrane potential does not switch off immediately once stimulated. Alternatively exposure to a high K medium may have reduced the relative permeability ratio P_{cat}/P_K so that the resting membrane potential returned to a value closer to the K equilibrium potential.

The extracellular K concentration always had to be increased to more than 100 mM to bring the resting potential close to zero, putting the intracellular concentration of the potential determining ions between 120 and 150 m-mole/l. cell H_2O, double the estimate obtained in cells isolated and maintained in Holtfreter solution (see p. 107) and within the range determined in whole *Xenopus* embryos at the late blastula stage of development (Slack *et al.* 1973). For the experiment of Fig. 9*A* the results can be fitted with the Goldman equation if $[K]_i$ is set at 145 mM and a^i_{Na} at 13 mM (Slack *et al.* 1973) and P_{cat}/P_K at the value of 0·09 given above. The line drawn through the points in Fig. 9*A* has been calculated for these values.

The intracellular sodium activities quoted here and on p. 107 assume that the intracellular Na distribution does not change when the cells are isolated. Slack *et al.* (1973) showed that in the intact embryo a large proportion of the total intracellular Na is sequestered out of the cytoplasm in an inactive form. At the late blastula stage Na_i (total) is about 40 m-mole/l. cell H_2O, whereas the Na activity is 13 or 14 mM. If Na were released from sequestering sites during the disaggregation procedure then the estimate of intracellular Na and K could be seriously in error. This is unlikely to be the case where the cell membrane potential is sensitive to the extracellular concentration of K, but might be a factor in those cells which do not respond to alterations in $[K]_0$.

Measurements of intercellular resistance in isolated pairs and clumps of cells

The spread of current from one cell to the next was monitored in small clumps of five to ten cells isolated in Holtfreter solution or Ringer solution, either by treatment of whole embryos with EDTA, or by micro-dissection from the inner surface of the vegetal pole. Individual cells, or groups of cells within a clump, could have high resting potentials yet be uncoupled from the remainder of cells in that group.

This finding contrasts with measurements on surface cells of the intact amphibian embryo, where all cells are coupled electrotonically by a low resistance intercellular pathway (Ito & Hori, 1966; Ito & Loewenstein, 1969; Palmer & Slack, 1970; Warner, 1973, 1974). The method of disaggregation was not the controlling factor; uncoupled cells were found in micro-dissected groups as well as in groups isolated in EDTA, although this does not rule out the possibility that some cells uncouple because of damage. Since all the groups of isolated cells came from the inner surface of the embryo, the present finding does not conflict with previous measurements made in cells on the external surface of the intact embryo.

Electrotonic coupling was measured in pairs of cells, isolated by micro-dissection in order to be certain that some of the junctions examined had been present in the intact embryo. Occasionally measurements were made on pairs isolated in EDTA; their behaviour was no different from the rest. Some of the pairs were formed by cell division in the bath after isolation; it is unlikely that any were formed by reaggregation. The electrode arrangement is drawn in Fig. 10. Two electrodes were put into one cell of the pair,

one to record the resting potential and monitor the voltage deflexion produced by current injected into the cell through the second micro-electrode. A third electrode was inserted into the adjacent cell to record the voltage change produced by current spreading through the junction. When possible a current-injecting electrode was inserted into cell 2 to measure the voltage decrement across the junction in both directions. Electrotonic potentials measured in this way from one cell pair are shown in Fig. 10*A*. The insertion of the fourth micro-electrode was not always successful and some measurements were obtained with three electrodes only. The input resistance of both cells in the pair and two values for the coupling ratio were determined and the results analysed using the equivalent circuit drawn in

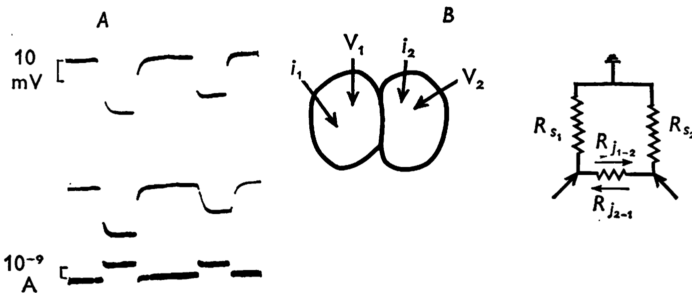

Fig. 10. Electrotonic coupling in cell pair mechanically isolated from late blastula. *A*, original records obtained from pair 14*B* (Table 2). In each case the upper trace gives the electrotonic potential recorded in the same cell as the current passing electrode. The middle trace gives the resultant voltage deflexion recorded in the adjacent cell and the lower trace the injected current pulses. *B*, diagram showing electrode arrangement and equivalent circuit used for analysis of results (Bennett, 1966).

Fig. 10*B* (Bennett, 1966), to give the surface resistance (R_s) and junctional resistance (R_j), assuming the leak resistance between the cells to the bath to be infinitely high. The high input resistance in most cells ($10^6\ \Omega$ or greater) and the generally good coupling ratios suggested that such leak currents were relatively small. Where measurements were made using three electrodes the surface resistance for both cells was assumed to be the same. The values calculated for all cell pairs examined are detailed in Table 2. When the input resistance for both cells of the pair is known, a value for R_j is calculated for each direction of current flow. These are given in Table 2 as R_{j1-2} and R_{j2-1}.

The results given in Table 2 show no difference between cells in Holtfreter solution and Ringer solution. The surface membrane resistances lie between 6×10^5 and $1 \times 10^9\ \Omega$ and the junctional resistances between 10^5 and $10^8\ \Omega$. Cells which are coupled electrotonically need not have the same resting potential, as illustrated earlier (see Fig. 9); a low resting potential

TABLE 2. Surface and junctional resistances in pairs of endoderm cells isolated from late blastula stages of *Xenopus laevis*

Pair no.	E_{m_1} (mV)	E_{m_2} (mV)	d_1 (μm)	d_2 (μm)	R_{s_1} (Ω)	R_{s_2} (Ω)	$R_{j_{1-2}}$ (Ω)	$R_{j_{2-1}}$ (Ω)	Method of isolation
					A. Holtfreter solution				
1	−10	−20	75	75	8×10^6	—	6×10^6	—	EDTA
2	−35	−40	75	75	$1\cdot9\times10^7$	—	8×10^6	—	Mechanical
3	−45	−45	100	100	$1\cdot6\times10^7$	—	$1\cdot2\times10^7$	—	Mechanical
4	−40	−40	70	70	9×10^6	—	No coupling	—	Mechanical
5	−24	−24	75	60	3×10^7	—	8×10^6	—	Mechanical
6	−32	−60	60	50	$1\cdot2\times10^7$	—	5×10^6	—	Mechanical
					B. Ringer solution				
1	−33	−18	80	80	$1\cdot25\times10^7$	—	$1\cdot1\times10^7$	—	Mechanical
2	−17	−15	130	130	$8\cdot3\times10^5$	—	$6\cdot3\times10^5$	—	Mechanical
3	−9	−15	150	100	2×10^7	—	$1\cdot44\times10^7$	—	EDTA
4	−40	−30	80	80	$1\cdot2\times10^7$	—	5×10^6	—	Mechanical
5	−14	−30	100	100	$9\cdot4\times10^6$	—	$4\cdot4\times10^5$	—	Mechanical
6	−8	−8	80	80	$2\cdot1\times10^7$	—	$1\cdot9\times10^7$	—	Mechanical
7	−64	−62	80	80	$1\cdot3\times10^7$	—	1×10^7	—	EDTA*
8	−38	−12	80	80	$1\cdot6\times10^7$	$1\cdot6\times10^6$	$1\cdot6\times10^6$	1×10^6	Mechanical
9	−30	−30	70	70	$2\cdot7\times10^7$	$2\cdot9\times10^7$	$2\cdot5\times10^7$	$2\cdot1\times10^7$	Mechanical
10	−13	−40	150	150	$2\cdot2\times10^6$	1×10^7	1×10^5	$1\cdot2\times10^5$	Mechanical
11	−14	−10	100	100	$2\cdot5\times10^7$	$1\cdot8\times10^7$	3×10^6	$3\cdot3\times10^6$	Mechanical
12	−14	−14	80	80	$1\cdot4\times10^7$	$1\cdot3\times10^7$	$1\cdot0\times10^7$	$1\cdot1\times10^7$	Mechanical
13	−10	−10	75	75	$7\cdot3\times10^6$	$6\cdot1\times10^6$	0	0	Mechanical†
14	−20	−25	70	70	$4\cdot6\times10^7$	$4\cdot6\times10^7$	$1\cdot5\times10^7$	$1\cdot5\times10^7$	Mechanical
15	−25	−18	100	80	$2\cdot5\times10^6$	$2\cdot6\times10^7$	$2\cdot4\times10^6$	$2\cdot2\times10^7$	Mechanical
16	−10	−10	125	175	$5\cdot6\times10^5$	$8\cdot8\times10^6$	$3\cdot5\times10^5$	$7\cdot3\times10^6$	Mechanical
17	−40	−40	80	80	1×10^9	$2\cdot5\times10^7$	$0\cdot5\times10^7$	15×10^7	Mechanical

* This cell pair illustrated in Fig. 9. † Unity coupling.

is not correlated with a low surface membrane resistance. In eight pairs the surface membrane resistance of both cells was closely similar and the resistance of the junction came to the same value for both directions of current flow. In another pair (8, Table 2*B*) the cells had dissimilar surface resistances, but the junctional resistance came to the same value in both calculations. These results suggest that the junction between many cells behaves as a linear resistive element.

On three occasions (pairs 15, 16 and 17, Table 2*B*) the value calculated for the junctional resistance depended on the direction of current flow; the surface resistance of the contacting cells also differed. Are these findings the result of some artifact or do they mean that the junctional resistance can vary according to the direction of current flow across it? This question cannot be answered without a detailed analysis of the voltage–current characteristics of each cell determined for current flow in both directions across the junction, as done by Furshpan & Potter (1959) when analysing the rectifying synapse of the crayfish giant axon.

Voltage–current plots were recorded in three cell pairs, but with three microelectrodes so that the input resistance of only one cell in the pair could be measured. Two pairs of cells showed no variation in coupling coefficient with voltage. The third showed complex voltage–current relations. In one cell the slope conductance fell as the cell was depolarized and the height of the electrotonic potential increased with time during a 1 sec current pulse. The associated cell showed a rise in slope conductance as the cell was depolarized; here the electrotonic potential fell with time during polarization. Either the voltage–current characteristics of the surface membrane of each cell rectified in opposite directions, or the properties of the junction interposed between them complicated the picture. Single, isolated cells showed only linear, or outwardly rectifying voltage–current relations, suggesting that such results were in part due to non-linear behaviour of the intercellular pathway.

In each of pairs 15, 16 and 17 (Table 2*B*) $R_{j_{2-1}}$ is greater than $R_{j_{1-2}}$. In 15 and 16 $R_{s2} > R_{s1}$, but in 17 $R_{s1} > R_{s2}$. This suggests that the difference between $R_{j_{2-1}}$ and $R_{j_{1-2}}$ does not result from a leak in one cell which gives an apparently rectifying junction because a greater proportion of current leaves via the leak for one direction of current flow.

DISCUSSION

The results described in this paper suggest that at the late blastula stage of development cells isolated from presumptive endoderm and mesoderm regions of the amphibian embryo form a heterogeneous population with respect to their membrane properties. This finding complements the grafting studies of Nieuwkoop (1969*a*, *b*) and confirms that morphological similarity between one blastula stage cell and another does not necessarily mean that all the cells are identical physiologically.

This conclusion assumes that the diversity of membrane response

observed is not produced by the procedures used to isolate the cells. It is difficult to argue firmly either in support or denial of this assumption. Variations in membrane potential and membrane resistance from one cell to the next might be the consequence of damage incurred during disaggregation or micro-electrode insertion, but the different absolute conductances of cations and anions found within the population seem less likely to arise from some non-specific effect of the isolation procedures, particularly since some cells are anion impermeable. The specific membrane resistances of almost all the cells examined were over 1000 Ω cm^2, which suggests that leaky cell membranes were not frequent occurrences. The different sensitivity of the membrane potential to alterations in extracellular K concentration of cells with closely similar initial membrane potentials also argues that the differences have some more specific basis. On balance it seems reasonable to conclude that the present finding of wide variations in membrane properties from one isolated cell to the next reflects, at least in part, the true properties of endoderm and mesoderm cells in the early amphibian embryo.

Some electrophysiological studies on single cells isolated from late morulae of *Triturus pyrrhogaster* have been reported by Ito & Loewenstein (1969) who recorded an average membrane potential of -29 mV in Holtfreter solution and -26 mV in Ringer solution. Both values fall within the range recorded here for cells of *Xenopus*. Ito & Loewenstein (1969) give a single value of 25×10^3 Ω cm^2 for the specific membrane resistance of one cell, which again falls within the range found in the present experiments. Their measurements on one pair of cells puts the junctional resistance at $\sim 10^7$ Ω. In the course of the present study a few measurements were made on cells isolated from blastula stages of *Triturus cristatus* which gave membrane resistances between 15×10^3 and 30×10^3 Ω cm^2. In accord with the present results Ito & Loewenstein (1969) found cells from *Triturus* isolated in a solution containing 80 mM-NaCl and 0·9 mM-KCl to have an average membrane potential of -2 mV in a solution with 61 mM-KCl.

Although the different membrane properties recorded here cannot be related to future fate until the properties of cells taken from a known location in the late blastula stage embryo, and therefore of known developmental fate, have been examined, it seems worth considering whether heterogeneity of membrane properties of the cells within the amphibian embryo has any implications for understanding the way in which spatial differentiation comes about. The membrane potentials of isolated cells vary widely, despite the similarity between different areas of the intact amphibian embryo (Slack & Wolpert, 1972), which implies that the properties of the individual cells are normally short circuited by current flow through the intercellular junction. Any change in properties of a cell, or group of cells would, therefore, give rise to a transient flow of ionic current between the cells until equilibrium is restored. The possibilities for cellular interactions on the basis of varying ionic permeabilities alone

therefore seem considerable. Many authors have proposed that the spatial pattern of differentiation is governed by the gradient of some substance or property from one cell to the next within the embryo (e.g. Child, 1941; Wolpert, 1969; Lawrence, Crick & Monroe, 1972), or by the propagation of signals from one or more 'pace-maker' regions in the embryo (Goodwin & Cohen, 1969). The molecule, or molecules, involved in the generation of such gradients or signals have yet to be identified, but the low resistance intercellular pathway has often been suggested as the channel for these interactions (e.g. Furshpan & Potter, 1968).

Electrophysiological experiments on whole embryos of a number of species have shown the ionic permeability of the intercellular junction to be high relative to the surface membrane conductance. The present measurements confirm the relatively high conductance of the intercellular pathway and suggest that the junctional membrane acts as a linear resistor to current flowing through it in the majority of cases. However in three pairs of cells the evidence suggested that the junctional membrane could discriminate between current flowing in one direction or the other across it. If the proportion of rectifying junctions at stage 8 is the same *in vivo* as *in vitro* then approximately 20 % of the cell to cell contacts in the whole embryo would be rectifying. The distribution of such junctions within the embryo could be of great importance for spatial patterning.

The area of membrane contact forming the junction is required to calculate the absolute ionic conductance of the junctional membrane. Without electromicrographs taken through the junctional regions of the cells studied electrophysiologically any value put upon the junctional conductance can only be a guess, but estimates can be made by assuming particular values for the contact area. The highest junctional resistance measured was $1{\cdot}5 \times 10^8\ \Omega$ (Table 2). Setting the contact area as a circle 1 μm in diameter gives a junctional conductance of 0·85 mho/cm^2, a circle diameter 0·1 μm would give a conductance of 85 mho/cm^2, and of 10 μm, $8{\cdot}5 \times 10^{-4}$ mho/cm^2. The smallest contact area gives a conductance which falls within the range of values calculated by Loewenstein, Socolar, Higashimo, Kanno & Davidson (1965) for the intercellular contacts between other electrotonically coupled cells. Table 2 shows the junctional resistance to lie between 10^5 and $10^8\ \Omega$; this could reflect either variations in contact area or junctional conductance from one cell pair to the next. Although the present results do not allow any conclusion to be drawn about the absolute permeability of the junctional membrane to small ions there is evidence that the permeability of the membrane between embryonic cells may be more restricted than in adult cells and organs. For example fluorescein (mol.wt. 332) does not pass from one cell to the next in pregastrula amphibian embryos (Slack & Palmer, 1969), starfish embryos

(Tupper & Saunders, 1972) and fish embryos (Bennett, 1973). EGTA (mol.wt. 384) also does not move across the intercellular junction in the early amphibian embryo (Baker & Warner, 1972).

The finding that cells in Holtfreter solution have the intracellular concentration of K, and probably other intracellular molecules, reduced to one half the value pertaining in the intact embryo deserves comment. Long term tissue culture of single cells or small aggregates isolated from early amphibian embryos has hitherto been done in media containing 60–80 mM-Na (Barth & Barth, 1959, 1963; Jones & Elsdale, 1963). If the intracellular concentrations of K and Na ions play a role in controlling the pattern of DNA synthesis, as considered by a number of authors (Kroeger & Lezzi, 1966; Kostellow & Morill, 1968; Slack *et al.* 1973), then a low K content of such isolated cells could influence both their subsequent pattern of differentiation and the way in which they respond to alterations in the composition of the culture media. For example the proportion of pigment cells in neuro-ectoderm cultures is noticeably higher in cells isolated in Holtfreter solution than in Ringer solution (P. A. Jackson, E. A. Messenger & A. E. Warner, unpublished). It therefore seems prudent to confirm findings based on cells isolated and cultured in low ionic strength media by culturing cells in media of higher ionic strength before drawing any general conclusions related to the normal processes of differentiation (cf. Barth & Barth, 1963).

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