Ion Levels and Membrane Potential in Chick Heart Tissue and Cultured Cells

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ABSTRACT Intracellular concentrations of sodium and potassium as well as resting potentials and overshoots have been determined in heart tissue from chick embryos aged 2–18 days. Intracellular potassium declined from 167 mM at day 2 to 117–119 mM at days 14–18. Intracellular sodium remained nearly constant at 30–35 mM during the same period. The mean resting potential increased from −61.8 mV at day 3 to about −80 mV at days 14–18. The mean overshoot during the same period increased from 12 to 30 mV. P_{Na}/P_K calculated from the ion data and resting potentials declined from 0.08 at day 3 to 0.01 at days 14–18. Thus, the development of embryonic chick heart during days 2–14 is characterized by a declining intracellular potassium concentration and an increasing resting potential and overshoot. Heart cells from 7- to 8-day embryos, cultured either in monolayer or reassociated into aggregates, were compared with intact tissue of the same age. The intracellular concentrations of sodium and potassium were similar in the three preparations and cultured cells responded to incubation in low potassium medium or treatment with ouabain in a manner similar to that of intact tissue. Resting potentials and overshoots were also similar in the three preparations.

INTRODUCTION

The past decade has seen an increased use of chick embryonic heart preparations both to study cardiac development and to investigate physiological processes common to all cardiac muscle. The electrical behavior of a variety of cardiac preparations has been studied, including intact tissue (Lieberman and Paes de Carvalho, 1965; Yeh and Hoffman, 1967, 1968) heart cells cultured in monolayers (Fänge, Persson, and Thesleff, 1956; Sperelakis, 1967; Lieberman, 1967; DeHaan and Gottlieb, 1968), and single cells reassociated into aggregates (McDonald, Sachs, and DeHaan, 1972; Sachs and DeHaan, 1973) or strands (Lieberman, Roggeveen, Purdy, and Johnson, 1972).

The ionic basis of electrical activity in embryonic chick heart muscle, at least at older stages, appears to be qualitatively similar to that described for

nerve (Hodgkin and Huxley, 1952) and adult cardiac tissue (Brady and Woodbury, 1960; Noble, 1966). However, both the resting potential and the action potential amplitude of the embryonic heart appear to increase with development. Meda and Feroni (1960) reported resting potentials of −35 mV in embryonic chick heart incubated for 37–67 h, while values between −60 and −72 mV have been recorded after 7 days of incubation (Lehmkuhl and Sperelakis, 1963; Lieberman, 1964). On the other hand, Yeh and Hoffman (1968) have reported that the resting potential does not increase significantly between days 5 and 19; the mean values recorded at these stages were −59.3 and −62.1 mV. More recently, Sperelakis and Shigenobu (1972) found that the mean resting potential was −70 mV at day 21 (hatching). These values are all somewhat lower than the −80 mV potentials recorded in adult chick ventricular tissue (Moore, 1965). The resting potential of embryonic rat heart tissue apparently does increase from a low value, about −35 mV at 10½ days of gestation, to its adult value of about −80 mV by birth (Couch, West, and Hoff, 1969). Thus it becomes necessary to reexamine the ontogeny of the membrane potential in chick embryo cardiac tissue.

Despite the widespread use of chick embryo heart preparations there is little data on the sodium and potassium levels in these tissues. Klein (1960) reported that the intracellular sodium concentration declined from 650 mM in 2-day hearts to about 50 mM in 19-day hearts, while the intracellular potassium concentration increased from 67 to 85 mM. In subsequent work Klein has attributed the high early sodium concentration to extracellular binding by cardiac jelly (Klein, 1963; Thureson-Klein and Klein, 1971). Harsch and Green (1963) reported that the intracellular sodium concentration increased from about 20 to 40 mM during days 6–18, while the intracellular potassium concentration declined from 160 to 90 mM during the same period. The data from Klein's 1960 study are frequently quoted in the literature, despite his own subsequent findings and the difficulty in reconciling this data with electrophysiological measurements (Krespi and Sleator, 1966; Yeh and Hoffman, 1967, 1968).

The present study was initiated to clarify and extend the relationship between ion levels and the membrane potential of embryonic chick heart tissue during development. In addition, we have compared ionic levels and electrical activity in cardiac tissue, tissue cultured cells, and cellular aggregates from 7- to 8-day chick embryos.

METHODS

General Procedures

Eggs from White Leghorn hens were incubated for 2–18 days. The embryos were removed from the eggs, decapitated, and their hearts dissected out in a pool of amniotic fluid. Hearts were trimmed of blood vessels and other extraneous tissue.

The preparation of cultures has been detailed previously (DeHaan, 1970). Hearts were dissociated into their component cells by the multiple-cycle trypsinzation method (DeHaan, 1967). Cells were plated in plastic culture dishes (Falcon Plastics, Oxnard, Calif., 150 mm diameter) at a density of 1 or 2 $\times$ 10^7 cells/plate. The cultures were incubated at 37°C under a water-saturated atmosphere of 95% air and 5% CO_2.

Heart cell aggregates were prepared after trypsin-dissociation of hearts into their component cells (McDonald, Sachs, and DeHaan, 1972; Sachs and DeHaan, 1973). An inoculum of 10^7 cells was added to 30 ml of 818B medium (DeHaan, 1970) contained in a 300 ml Erlenmeyer flask. The flask was gassed with 5% CO_2, 10% O_2, 85% N_2, sealed with a silicone stopper, and placed in a gyratory shaker bath (37°C) at 70 rpm for 18–24 h.

Determination of Sodium and Potassium Content

Samples of cardiac tissue weighing between 2 and 10 mg were used for ion content analysis. Samples from 11-, 14-, and 18-day embryos consisted of a strip of ventricle, at 7 days the sample included both ventricles, and at 5 days most samples contained the trimmed ventricles of two hearts. When whole ventricles were used, they were slit down the midline to the apex to expose both epicardial and endocardial surfaces to the medium. Samples from 3-day and 2- to 2½-day embryos averaged 12 and 20 whole hearts per sample, respectively. Tissues were incubated at 37°C for 10–15 min in physiological salt solution equilibrated with 95% O_2 and 5% CO_2. Tissues from embryos aged 5–18 days were dipped in isotonic choline chloride solution (2.26%) for 2 s, blotted three times between lens paper, and weighed in tared low-alkali test tubes (Thermal American Fused Quartz Co., Montville, N. J.). Tissues from embryos aged 2–3 days were suspended in about 0.4 ml of medium and transferred to Intramedic polyethylene luer-end catheters (Clay-Adams, Inc., Parsippany, N. J.) widened at one end to form an effective reservoir. By heat-sealing the tube approximately 5 cm below the flared portion a functional small-bore plastic centrifuge tube was formed. After the addition of the tissue suspension to the tube, it was centrifuged at 1500 *g* for 3 min. The supernatant was quickly aspirated leaving a columnar tissue pellet of 0.4–0.8 mm in height. The sealed base of the catheter tube was cut off with a clean razor blade and the tissue pellet extruded (with a stainless steel wire) into a tared low-alkali test tube and weighed.

Tissue cultured cells, which had been incubated for 18–24 h in 818B medium were removed from the plastic dish by a 10 min exposure to serum-free, calcium-magnesium-free medium containing 0.15% trypsin. After trypsin inactivation by resuspension in fresh 818B medium, cells were briefly centrifuged and resuspended at about 10^7 cells/ml. About 0.4 ml of this suspension was transferred to a catheter-centrifuge tube and spun at 1200 *g* for 3 min. The supernatant was quickly aspirated, the tip of the tube cut off, and the cell pellet extruded into a tared low-alkali test tube and weighed.

Heart cells were reaggreated in 818B medium for 18–24 h. Aggregates were concentrated in suspension after a brief centrifugation, and a suitable portion transferred

to a catheter-centrifuge tube for centrifugation at 1200 *g* for 3 min. The supernatant was quickly aspirated, the tip of the tube cut off, and the aggregate pellet extruded into a tared low-alkali test tube and weighed.

Tissue and pellets were digested with concentrated HNO_3 in a heated aluminum block. After the acid had evaporated, the residue was dissolved in a suitable volume of lithium chloride diluent (15 mM) and sodium and potassium were determined simultaneously on a digital read-out flame photometer (Instrumentation Laboratory, Inc., Lexington, Mass., Model 143).

Water Content

Blotted tissues or cell and aggregate pellets prepared as above were weighed on tared cover slips and dried for 12 h in an oven at 110°C. After cooling in a desiccator, the dry weight of the residue was determined and the water content expressed as the difference between wet weight and dry weight.

Determination of Extracellular Space (ECS)

The extracellular space was determined with [^{14}C]inulin (New England Nuclear Corp., Boston, Mass.). The radioactivity of the medium was about 0.15 μCi/ml corresponding to an inulin concentration of 0.3 mg/ml. As a result of preliminary experiments and the calculations of Ling and Kromash (1967) on the diffusion of inulin into the extracellular space of thin muscle sheets, tissues from embryos aged 5–18 days were incubated in [^{14}C]inulin medium for 1 h, dipped in isotonic choline chloride solution for 2 s, and blotted between lens paper. The tissues were placed in tared 5-dram sample vials, weighed, and solubilized in 0.8 ml of Soluene 100 (Packard Instrument Co., Inc., Downers Grove, Ill.). The solution was transferred to scintillation vials, and the sample vials were washed with two 1 ml portions of Bray's solution (Bray, 1960). An additional 10 ml of Bray's solution was added to each vial and ^{14}C counted on a Packard Tri-Carb Scintillation counter.

Tissues from 2- to 3-day embryos were incubated in [^{14}C]inulin medium for 30 min; cells and aggregates were incubated for 15 min. After centrifugation, the supernatant was saved to determine specific activity. The pellets were extruded into tared 5-dram sample vials, weighed, and treated as described above.

Electrophysiology

Hearts from embryos aged 5–18 days were pinned to a layer of paraffin in 35 mm Falcon plastic tissue culture dishes (Falcon Plastics, Oxnard, Calif.), while hearts from 3-day embryos were studied *in situ*, the embryo being pinned to the paraffin. All hearts were incubated in either physiological salt solution or 818B; cells and aggregates were incubated in 818B. Cells were examined 24 h after plating while aggregates were examined 3–6 h after plating. The culture dish was mounted on the warm stage (37°C) of an inverted phase-contrast microscope. A gassing ring directed a stream of 95% O_2-5% CO_2 (hearts) or 10% O_2-80% N_2-10% CO_2 (cells and aggregates) over the dish. Mineral oil was layered over the medium in the dish to prevent evaporation (DeHaan and Gottlieb, 1968).

Transmembrane potentials were recorded between intra- and extracellular micro-

pipette electrodes. Both electrodes were filled by the glass-fiber procedure of Tasaki et al. (1968), the intracellular, high impedance pipette with 2 M KCl, and the extracellular, low impedance pipette with an isotonic balanced salt solution. The tip potentials of a series of 32 microelectrodes of 20–180 MΩ resistance were measured by comparing the electrode potential before and after breaking the electrode tip. None had tip potentials greater than 6 mV, nor was tip potential correlated with resistance. For the present study, intracellular microelectrodes were selected with resistances of 30–100 MΩ. The intracellular electrode was connected through an Ag-AgCl electrode holder to a capacitance-compensated unity-gain amplifier ELSA-4, Electronics for Life Sciences, Rockville, Md.) and the indifferent (extracellular) to ground through a 50 mV calibrator and a similar holder. The membrane potential was displayed on a Tektronix 502A oscilloscope (Tektronix, Inc., Beaverton, Oreg.) and stored as required on a Tektronix 5103/D13 storage oscilloscope.

Solutions

The composition of the physiological salt solution was (millimoles/liter): NaCl 116.0, KCl 4.5, $MgSO_4 \cdot 7H_2O$ 0.8, $NaH_2PO_4 \cdot H_2O$ 0.9, $CaCl_2 \cdot 2H_2O$ 1.8, $NaHCO_3$ 26.2, and glucose 5.5. Medium 818B has been described previously (DeHaan, 1970). It contained 20% M 199 (Grand Island Biological Co., Grand Island, N. Y.), 2% heat-inactivated horse serum (Colorado Serum Co., Denver, Colo.), 4% heat-inactivated fetal calf serum (Grand Island Biological Co.), and 4% chick embryo extract. Both the fetal calf serum and the chick embryo extract were previously dialyzed against potassium-free physiological salt solution. The antibiotics present were pencillin-G-sodium (100 U/ml) and streptomycin sulfate 0.05 mg/ml; the medium was brought to volume with potassium-free salt solution. The potassium concentration of the medium was adjusted to 4.5 mM by the addition of salt solution containing 100 mM KCl.

RESULTS

Ion Determinations

The results of determinations on freshly dissected ventricles from chick embryos aged 5–18 days, analyzed for water, sodium, and potassium content are shown in Table I (columns 1–4). Tissue water content declined slightly with increasing age, from 86.4 ml/100 g for 5-day hearts to 83.0 ml/100 g for 18-day hearts. Total sodium content generally decreased with increasing age, ranging from 68.0 mM/kg wet weight for 5-day hearts to 58.6 mM/kg wet weight for 18-day hearts. Similarly, the potassium content decreased with increasing age, declining from 78.3 mM/kg wet weight for 5-day hearts to 64.4 mM/kg wet weight for 18-day hearts.

In addition to total water and ion content, a determination of the extracellular space is required to calculate intracellular ion concentrations. These values are shown in Table I (column 5). It is well recognized that such determinations are fraught with difficulties (Ling and Kromash, 1967;

TABLE I

THE SODIUM, POTASSIUM, WATER CONTENT, AND EXTRACELLULAR SPACE ([^{14}C]INULIN) OF CHICK EMBRYO VENTRICULAR TISSUE*

(1)	(2)	(3)	(4)	(5)
		Ion content		
Embryo age	H_2O	Na	K	Extracellular space
days	*ml/100 g*	*mM/kg wet wt.*		*ml/100 g wet wt.*
5	86.4 ± 0.3 (7)‡	68.0 ± 2.2	78.3 ± 2.2	34.9 ± 2.0 (7)
7	86.0 ± 0.4 (12)	62.4 ± 1.6	78.2 ± 1.2	33.5 ± 1.0 (15)
11	84.6 ± 0.4 (12)	63.5 ± 1.6	72.6 ± 2.1	30.1 ± 0.8 (8)
14	83.8 ± 0.3 (12)	61.6 ± 0.9	66.6 ± 1.6	27.5 ± 1.3 (8)
18	83.0 ± 0.3 (12)	58.6 ± 1.6	64.4 ± 0.8	27.4 ± 0.8 (26)

* Values are mean ± standard deviation.

‡ Number of determinations for data in columns 2–4.

Vick, Hazelwood, and Nichols, 1970), and that inulin is an imperfect extracellular marker, since it may not distribute uniformly in the extracellular volume (Page, 1965). Nevertheless, [^{14}C]inulin was utilized in this study because of its widespread use by others as an indicator of extracellular space in such muscle tissue as uterine smooth muscle (Taylor, Paton, and Daniel, 1970), cat papillary muscle (Page and Storm, 1965), guinea pig atria (Glitsch, 1969), and guinea pig ventricular muscle (McDonald and MacLeod, 1971), and because it is one of the few markers to which early cardiac cells are impermeable (Guidotti et al., 1968).

Experiments were conducted to determine the effect of incubation time and tissue mass on the measured extracellular space of embryonic ventricular tissue. Ventricular tissue from 18-day chick embryos was incubated in medium containing approximately 0.15 μCi/ml [^{14}C]inulin for 30, 60, and 90 min (six to nine measurements each). Values were, respectively, 20.1 ± 0.9, 26.5 ± 1.7, and 27.5 ± 1.9 ml/100 g. Thus the space occupied by inulin appeared to reach equilibrium within 60 min of incubation, the increase during the next 30 min being negligible. Extracellular space in all further experiments on 5- to 18-day tissue was therefore measured after a 60 min incubation with [^{14}C]inulin medium.

Measurements of ECS were also made on single and double strips of 7- and 18-day ventricle to ensure that any extracellular space differences between hearts of different ages did not result from the smaller size of the preparations from the younger hearts. The measured values of extracellular space were found to be the same in 18-day preparations weighing between 2 and 5 mg as in those weighing between 10 and 20 mg. Similar results were obtained whether the sample contained a single ventricular strip or two such strips. The extracellular space of 7-day ventricular strips was also independent of tissue mass or number of strips per sample.

The hearts of chick embryos younger than 5 days are too small and delicate to be handled by the methods used for the older hearts. At 3 days the heart is of a tubular form, being approximately 2 mm in length, 0.5 mm in diameter, and having a wall approximately three cells deep. Groups of hearts could therefore be spun down into a semi-solid pellet and analyzed as described under Methods. Extracellular space in this pellet was determined after previous incubation of the hearts for 30 min in [^{14}C]inulin medium. Additional samples incubated for 75 min did not differ significantly from the 30-min samples. The extracellular space, total pellet water, and sodium and potassium content of pellets from 2- to 2½ and 3-day old hearts are shown in Table II. As mentioned above, the pellet formed after centrifugation of

TABLE II

THE EXTRACELLULAR SPACE ([^{14}C]INULIN) AND THE TOTAL SODIUM, POTASSIUM, AND WATER IN PELLETS OF HEARTS FROM 2- TO 3-DAY CHICK EMBRYOS

Embryo age	Number	ECS	H_2O	Ions	
				Na	K
days		*ml/100 g*	*ml/100 g*	*mM/kg wet wt.*	
2–2½	8 (20)*	49.1 ± 1.6	89.1 ± 0.4	83.4 ± 2.8	68.9 ± 1.2
3	9 (12)*	52.8 ± 1.4	91.1 ± 0.3	85.4 ± 1.5	62.8 ± 1.5

* Average number of hearts per sample.

these samples was not of a solid nature and included both the intercellular tissue space and presumably a substantial inter-heart space. This is reflected in the large extracellular space of 49.1–52.8 ml/100 g and water content of 89.1–91.1 ml/100 g, values which are therefore not directly comparable to those listed in Table I (column 5).

The intracellular concentration of sodium and potassium was calculated using the mean values of extracellular space, water content, and sodium and potassium content after suitable correction for ions trapped in the extracellular space. In addition, cell water content was calculated as a percentage of cell wet weight. The results are presented in Fig. 1. Cell water and intracellular sodium concentration did not change to any great degree from day 2 through day 18. However, the intracellular potassium concentration declined from 167 mM/kg cell water in 2- to 2½-day hearts to 117 mM in 14-day hearts.

A further aim of this study was to compare the intracellular concentrations of sodium and potassium in chick embryo heart tissue with those of heart cells cultured in monolayer or associated into aggregates by rotation. Since most studies on tissue cultured heart cells have been done with cells from 7-

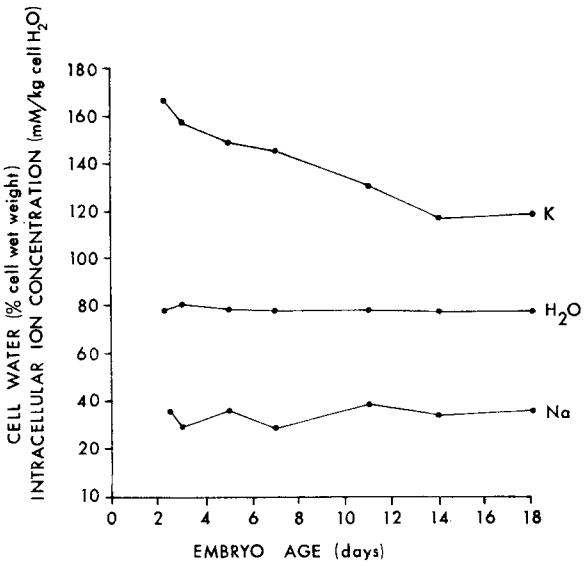

FIGURE 1. The intracellular concentrations of sodium and potassium and the cell water content of embryonic chick heart muscle. Intracellular ion concentrations were calculated using the mean ion and water contents and mean extracellular space values presented in Tables I and II. Cell water was calculated as a percentage of cell wet weight using the mean tissue water content and extracellular spaces.

to 8-day embryos, this age was chosen for the comparison. Table III presents the extracellular space (fluid trapped in the cell pellet), water content, total ion content, and mean intracellular sodium and potassium concentration of cells under three circumstances: (*a*) immediately after trypsinizing from the plate, (*b*) after a 1 h recovery in suspension, and (*c*) reassociated into aggregates ranging in volume from 5×10^4 to 1×10^6 μm^3, each aggregate composed of 10^2–10^3 cells.

The extracellular space and water content of the isolated heart cell pellets did not change significantly during the 1 h recovery period, nor did ion content; there was, however, a loss of sodium and a gain of potassium. Intracellular sodium concentration declined from 60.3 to 40.2 mM while intracellular potassium increased from 118.4 to 139.2 mM. The net movement of ions was therefore quite comparable suggesting a 1:1 coupling of sodium and potassium transport during this recovery period. Three samples were allowed to recover in suspension for a 2 h period but there was no further change in ion content. The intracellular sodium concentration of 33.1 mM and potassium concentration of 146.1 mM measured in aggregates (Table III, column 3) are in better agreement with the ion levels found in 7 day intact tissue (Fig. 1) than in 7- to 8-day monolayer cultured cells even after the 1 h recovery period (Table III, column 2).

TABLE III

THE EXTRACELLULAR SPACE ([^{14}C]INULIN) TOTAL SODIUM, POTASSIUM AND WATER, AND THE INTRACELLULAR CONCENTRATIONS OF SODIUM AND POTASSIUM IN PELLETS OF TISSUE CULTURED HEART CELLS FROM 7- TO 8-DAY CHICK EMBRYOS, AND FROM AGGREGATES PREPARED FROM SUCH CELLS

	After trypsin*	1-hr Suspension‡	Aggregates§
ECS, *ml/100 g*	23.2 ± 1.0	23.4 ± 1.1	20.4 ± 1.0
H_2O, *ml/100 g*	84.7 ± 0.4	84.0 ± 0.4	84.3 ± 0.4
Na_T, *mM/kg wet wt.*	69.6 ± 1.7	57.5 ± 1.5	50.1 ± 3.0
K_T, *mM/kg wet wt.*	73.3 ± 1.1	84.6 ± 1.5	94.5 ± 1.2
Na_i, *mM/kg cell* H_2O	60.3	40.2	33.5
K_i, *mM/kg cell* H_2O	118.4	139.2	146.1
No. of determinations	13	10	7

* Determinations performed on pellets of cells immediately upon removal from the plate.

‡ Determinations performed 1 h after trypsinization, during which time cells were suspended in $818B_{4.5}$ medium.

§ Pellets contained 100–200 aggregates taken directly from the gyratory flask. Na_T and K_T refer to pellet ion content and are expressed as millimoles/liter per kilogram pellet wet weight. Na_i and K_i are the calculated intracellular concentrations (millimoles/liter per kilogram cell water) based on mean ion contents. Values are mean ± SE.

It was of interest to compare the values obtained for cultured chick heart cells with those of other cell types grown in culture. The comparison is shown in Table IV; it can be seen that the present results are in good agreement with the published data on other cell types.

The response of 8-day cultured cells to low potassium medium and to ouabain was compared to the response of 8-day heart tissue subjected to the same treatment. Cells and hearts were incubated for 1 h in medium containing 1 mM potassium, 4.5 mM potassium with 10^{-5} M ouabain, or 4.5 mM potassium with 10^{-4} M ouabain. The results are summarized in Table V. Control values were determined immediately after tissue dissection or cell trypsinization. At this time, hearts had lower sodium and higher potassium concentrations than cells. However, after incubation for 1 h in 4.5 mM potassium medium the ion levels in hearts and cultured cells were not significantly different. When hearts were incubated in 1 mM potassium medium they gained 9 mM sodium and lost 7.5 mM potassium. Cultured cells recovering in 1 mM potassium medium did not lose sodium and gain potassium to the same degree as cells recovering in 4.5 mM potassium; the net increase in intracellular potassium was 4.1 mM vs. 23 mM during recovery in 4.5 mM potassium. These movements of potassium in response to incubation in low potassium medium are similar to those reported for frog heart by Gadsby, Niedergerke, and Page (1971).

As expected, ouabain caused a net loss of potassium and gain of sodium in both hearts and cultured cells. The dose necessary to produce these downhill

TABLE IV

COMPARISON OF INTRACELLULAR SODIUM AND POTASSIUM IN CULTURED CELLS

Cell line	Ions		References
	Na	K	
	mM/l cell H_2O		
Chick heart	40	139	Present study
Baby hamster kidney	28	156	McDonald et al. (1972)
HeLa	39	135	Morrill and Robbins (1967)
Ehrlich ascites tumor	—	130–143	Bittner and Heinz (1963)
Mouse leukemic lymphoblast	19	136	Jung and Rothstein (1967)
Sarcoma-180	—	135	Lubin (1967)
Mouse L cells	9	167	Lamb and Mackinnon (1971)

TABLE V

THE EFFECT OF LOW POTASSIUM MEDIUM AND OUABAIN ON THE INTRACELLULAR CONCENTRATIONS OF SODIUM AND POTASSIUM IN 8-DAY CHICK EMBRYO HEART AND TISSUE CULTURED CELLS

Experimental conditions	Heart		Tissue culture	
	Na_i	K_i	Na_i	K_i
Control	33.7 ± 3.0	138.5 ± 3.9	62.0 ± 2.1	118.5 ± 2.8
1 h 4.5 mM K	28.7 ± 5.0	142.9 ± 3.1	34.3 ± 1.9	141.5 ± 3.0
1 h 1 mM K	42.8 ± 3.1	131.3 ± 4.1	58.6 ± 2.5	122.6 ± 2.8
1 h ouabain 10^{-5} M	38.9 ± 2.1	138.0 ± 1.5	52.7 ± 2.4	125.3 ± 2.6
1 h ouabain 10^{-4} M	70.8 ± 2.1	98.6 ± 2.3	98.0 ± 2.9	79.6 ± 2.7

Intracellular sodium (Na_i) and potassium (K_i) concentrations are millimoles/liter per kilogram cell water. Control refers to determinations immediately after dissection (hearts) or trypsinization (cells). Treatments of tissue cultured cells were carried out during the suspension period after trypsinization. Values are mean ± SE (n = 4).

movements in the intact heart was in excess of 10^{-5} M whereas the same dose largely prevented the ion changes in culture cells during the recovery period. Hearts treated for 1 h with 10^{-4} M ouabain gained 37.1 mM sodium and lost 30.9 mM potassium; cultured cells gained 36 mM sodium and lost 38.9 mM potassium. It can be concluded that whole tissue and cultured cells responded nearly identically to treatment with 10^{-4} M ouabain and in both cases the gain of sodium was closely paralleled by the loss of potassium.

Membrane Potentials

Membrane potentials were measured in ventricular tissue from chick embryos aged 3–18 days. Typical recordings obtained at 3, 7, and 18 days are shown in Fig. 2. It is apparent that the resting potential and overshoot increased with embryo age. The 3 day heart was beating at about 170/min

while the older hearts were beating at 60/min. Mean resting potentials and overshoots, as a function of embryo age, are shown in Fig. 3. Between days 3 and 14 the resting potential increased from −61.8 to −79.5 mV while the overshoot increased from 12.2 to 30.0 mV. There was no significant increase

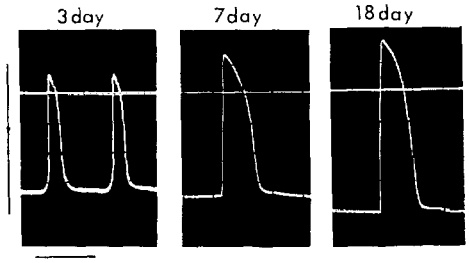

FIGURE 2. Typical ventricular action potentials from chick embryos aged 3, 7, and 18 days. Note that the 3 day heart was beating at about 170/min while the 7-day and 18-day hearts were beating at about 60/min. Vertical calibration represents 100 mV; horizontal calibration represents 400 ms.

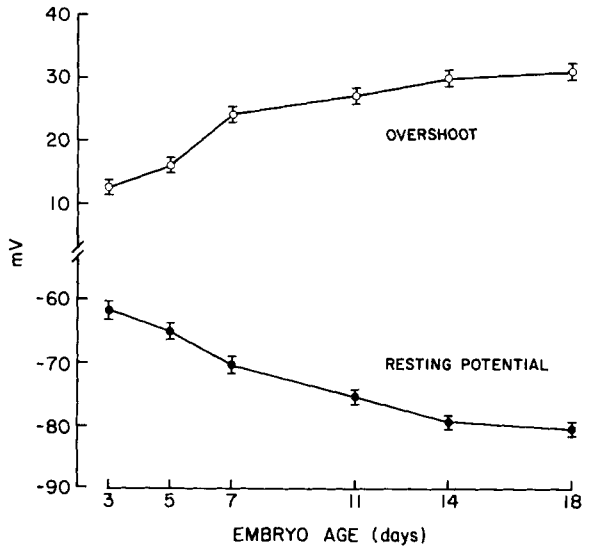

FIGURE 3. The resting potential and overshoot of ventricular tissue as a function of embryo age. The values are mean ± SE based on 45–65 penetrations in four to seven hearts at each embryo age.

between days 14 and 18. The data are based on 45–65 penetrations in four to seven hearts at each embryo age. Although the scatter was slightly greater in tissue from 3- and 5-day embryos there can be little doubt about the trend. On day 5 there were no resting potentials more negative than −80 mV and no overshoots greater than 30 mV. By day 14, more than 50% of the resting

potentials were between −80 and −90 mV, and 50% of the overshoots were between 30 and 45 mV. The mean values of the peak potentials in each heart at the various ages were compared. Between days 5 and 14, the mean peak resting potential increased significantly ($P<0.01$) from −74.9 to −85.9 mV while the overshoot increasee significantly ($P<0.01$) from 25.4 to 40.1 mV.

The threshold concentration of external potassium required to block pulsatile activity in chick embryonic heart cells declines with increasing embryo age (DeHaan, 1967, 1970). This has led to speculation that the permselectivity of the membrane changes with development (DeHaan, 1970). Yeh and Hoffman (1968) suggested that the resting potential was closer to the potassium equilibrium potential, E_K, at 19 days than at 6 days. Both of these predictions appear to have been correct. Table VI shows the mean resting

TABLE VI

THE MEASURED RESTING POTENTIAL AND THE CALCULATED POTASSIUM EQUILIBRIUM POTENTIAL AND SODIUM/POTASSIUM PERMEABILITY RATIO OF EMBRYONIC CHICK HEART MUSCLE

	Embryo age (days)					
	3	5	7	11	14	18
E_m	−61.8	−64.7	−70.6	−75.5	−79.5	−80.3
E_K	−94.2	−92.7	−92.2	−89.2	−86.3	−86.0
P_{Na}/P_K	0.078	0.062	0.041	0.022	0.010	0.009

E_K was calculated from the Nernst equation $E_K = RT/F \ln K_i/K_o$ using the mean values for intracellular potassium concentration. P_{Na}/P_K was calculated from the Goldman equation,

$$E = RT \ln \frac{K_o + P_{Na}/P_K(Na_o)}{K_i + P_{Na}/P_K(Na_i)},$$

using the mean values for resting potential and intracellular concentrations of sodium and potassium.

potential (E_M) and the calculated E_K and P_{Na}/P_K (utilizing the ion data and the Goldman equation at each embryo age). The difference between E_M and E_K decreased from 32.4 mV at 3 days to 5.7 mV at 18 days. The convergence of E_M and E_K was reflected in the decline of P_{Na}/P_K from 0.078 at 3 days to 0.009 at 18 days.

A comparison of the resting potentials and overshoots measured in 7- to 8-day chick embryo heart tissue, monolayer tissue cultured cells and heart cell aggregates is shown in Table VII. Utilizing E_M measurements and the ion data, E_K, E_{Na} and P_{Na}/P_K have been calculated for the three preparations. E_M ranged from −68.4 mV in monolayer tissue cultured cells to −70.7 mV in the intact tissue. The overshoots were also in good agreement, ranging from 22.0 mV in tissue cultured cells to 25.9 mV in the aggregates. As a result of the agreement between membrane potentials and ion levels in the three

TABLE VII

MEMBRANE POTENTIALS AND IONIC CORRELATES OF 7- TO 8-DAY CHICK EMBRYO HEART PREPARATIONS

	Intact tissue	Monolayer tissue culture	Aggregates
E_M , mV	−70.7 ± 0.7 (70)	−68.4 ± 1.2 (30)	−69.9 ± 1.2 (24)
Overshoot, mV	24.4 ± 0.6 (70)	22.0 ± 0.8 (30)	25.9 ± 0.8 (24)
E_K , mV	−91.5	−90.9	−92.2
E_{Na} , mV	40.3	33.7	37.0
P_{Na}/P_K	0.039	0.044	0.043

E_K and E_{Na} were calculated from the Nernst equation using the mean intracellular concentrations of sodium and potassium. P_{Na}/P_K was calculated from the Goldman equation using the mean values for resting potential (E_M) and intracellular ion concentrations. In the case of pacemaker-type cells E_M was taken as the maximum diastolic potential. E_M and overshoot values are mean ± SE, number of determinations in parentheses.

preparations, the calculated values of E_K , E_{Na} , and P_{Na}/P_K were also quite comparable.

DISCUSSION

Intracellular Sodium and Potassium

The electrolyte content of chick embryo heart tissue as measured by Klein (1960) differed significantly from the data of Harsch and Green (1963). Klein's value for early intracellular potassium (67 mM) appears to be quite low while that for intracellular sodium (650 mM) is extremely high. No mention was made of extracellular space measurements in Klein's 1960 report. In more recent experiments the bulk of this sodium has been attributed to binding by cardiac jelly, based on isotope exchange techniques (Klein, 1963) and histochemical staining with potassium pyroantimonate (Thureson-Klein and Klein, 1971).

The present findings are difficult to reconcile with Klein's measurements. We made no attempt to remove cardiac jelly from the younger hearts and yet, as shown in Table II, the total sodium content of 3-day hearts was about 85 mM/kg heart pellet. By day 5 any contribution by sodium bound to cardiac jelly would be insignificant (Thureson-Klein and Klein, 1971), leaving unexplained the difference between our sodium values at this age and Klein's. The intracellular sodium values in the present study, between 30 and 40 mM, are, however, compatible with values reported from adult cardiac muscle (Page and Storm, 1965; McDonald and MacLeod, 1971).

Harsch and Green's (1963) determinations on ventricular tissue from 8-, 11-, and 18-day embryos indicated that intracellular potassium declined from about 145 mM in 8-day preparations to 95 mM in 18-day preparations.

During the same period intracellular sodium remained relatively constant, increasing from about 28 mM at 8 days to about 38 mM at 18 days. With the exception of the lower potassium values they measured in 18-day preparations, their results are in close agreement with our own.

The results reported here are dependent on the validity of the measurement of extracellular space with [^{14}C]inulin, for it is on the basis of such measurements that intracellular water—and therefore intracellular ion concentrations—are calculated. Aside from the fact that inulin may not distribute evenly in the extracellular space of mature tissue, we face the added problem that membrane permeability to nonelectrolytes may change with development. Guidotti and Foa (1961), for example, have shown that sorbitol, which is restricted to the extracellular space in adult heart tissue, equilibrates in less than 10 min with 90% of the tissue water in the 5 day embryonic heart. On the other hand, they have also shown that inulin does not share this high permeability with sorbitol (Guidotti, et al., 1968). Our own data indicate that measured inulin space does not increase appreciably after 60 min in 18-day tissue or after 30 min in 2-3 day tissue, and at all ages yields calculated values of cell water that remain constant at about 80% of cell wet weight. This would not be the case if younger hearts were markedly more permeable to inulin than older hearts.

Our ECS value for ventricular tissue from 5-day embryos was 34.9 ml/100 g wet weight (Table I, column 5). This may be compared to the value of 35.0 ml/100 g in 5-day chick hearts reported by Guidotti et al. (1968). Values from 7 day tissue were not significantly different, but by 11 days the extracellular space had declined to 30.1 ml/100 g and by 14 days to 27.5 ml/100 g. No difference was noted between 14 and 18 days. A decline in extracellular space with development has been noted in chick embryonic skeletal muscle (Boethius and Knutsson, 1970); a similar, though steeper fall in the inulin space was measured in embryonic chick heart by Harsch and Green (1963).

Membrane Potentials

The resting potential in developing chick thigh muscle has been reported to increase from about −30 mV at day 3 to about −65 mV at day 19 (Boethius and Knutsson, 1970). Similarly, the resting potential of embryonic rat heart increased with development, from about −35 mV at 10½ days to about −80 mV at 20 days (Couch et al., 1969). In the present study, the resting potential of embryonic chick ventricular tissue increased from −61.8 mV at day 3 to −80.3 mV at day 18. It is of interest to note that the rat embryonic heart first begins spontaneous beating on day 10 and that of the chick on day 2, yet both gestational periods are about 21 days long. Preliminary measurements on 2-day old chick hearts indicate that the resting

potential is about −40 to −55 mV with overshoots in the range 5–15 mV (McDonald and Sachs, unpublished observations).

It is difficult to ascertain the extent to which factors such as tissue morphology and mechanical activity influenced the measured resting potentials. Generally speaking it was more difficult to secure long-lasting impalements in the 3-day heart than in the others. However, the "impaleability" of the 5 and 7 day tissue was judged to be as good as the 14 and 18 day tissue. Since cell injury caused by microelectrode penetration can lead to membrane leakiness and resultant lowering of the observed resting potential, it might be argued that the increase in resting potential with age was merely a function of increased fiber diameter resulting in a reduced injury potential. Although the cell fiber diameter in intact hearts at each embryo age has not been measured directly, isolated cells in culture from embryos aged 3–14 days do not exhibit substantial differences in size. Moreover, neither the fiber diameter of rat embryonic ventricular tissue (Couch et al., 1969) nor of embryonic chick thigh muscle has been seen to increase during the period of striking increase in resting potential (Boethius and Knutsson, 1970).

Klein (1961) suggested that the cation transport pump in embryonic heart becomes increasingly effective with age. Couch et al. (1969) raised the possibility that such an increase in pump activity with age might lead to an increased intracellular concentration of potassium and a gradually increasing resting potential. The present study indicates that intracellular potassium declines during the period of increasing resting potential. A plausible explanation for these changes is that the P_{Na}/P_K of the membrane undergoes a gradual reduction during development. The P_{Na}/P_K declined from 0.078 at 3 days to 0.01 at 14 days. These values may be compared with those of Sperelakis and Shigenobu (1972). Using a different technique (E_M vs. K_o) they concluded that P_{Na}/P_K declined from 0.2 at 3 days to 0.01-0.05 at 15 days. In addition, it is of interest to note that the P_{Na}/P_K of adult cat cardiac muscle is approximately 0.01 (Page, 1962).

The increase in action potential overshoot observed here with embryo age (Fig. 3) corresponds with the findings in embryonic rat heart (Couch et al., 1969). Yeh and Hoffman (1968) manipulated the resting potential of embryonic chick ventricular tissue by varying the external potassium concentration, and noted that the magnitude of the overshoot was dependent on the resting potential level. The availability of the sodium-carrying system is dependent on resting potential in nerve (Hodgkin and Huxley, 1952) and adult cardiac muscle (Weidmann, 1955; Beeler and Reuter, 1970). If embryonic chick cardiac muscle behaves in a similar manner (Yeh and Hoffman, 1968), the increasing overshoot with development may be related to the increasing resting potential.

Although heart muscle from both young and old chick embryos is inexcitable in sodium-free medium (Shigenobu and Sperelakis, 1971; Pappano, 1972) it is clear that the ionic mechanisms underlying the rising phase of the action potential undergo changes during development. Hearts from embryos aged 2–4 days continue beating in tetrodotoxin (TTX) 10^{-5} g/ml while by day 7 spontaneous activity is blocked by TTX 10^{-7} g/ml (McDonald et al., 1972). Further, the maximum rate of rise of the action potential increases from 10–20 V/sec at days 2 to 4, to about 90 V/sec at day 7 and 160 V/sec at day 18 (Sperelakis and Shigenobu, 1972; Sachs, McDonald, and DeHaan, in preparation). These findings suggest a transition from "slow" channels to "fast" channels (see Paes de Carvalho, Hoffman, and de Paula Carvalho, 1969; McDonald et al., 1972), and it is possible that the increasing overshoot with age is related to this channel transition.

Intact Tissue, Cultured Cells, and Aggregates

The usefulness of tissue cultured heart cells depends in part on whether they are physiologically similar to the cells composing their parent tissue. To our knowledge, the measurement of sodium and potassium in tissue cultured heart cells has been attempted only once previously, by Burrows and Lamb (1962). Their analysis of chick embryonic heart cell cultures which had been overgrown by fibroblasts yielded values of 16 mM sodium and 186 mM potassium. In contrast, up to 50% of the monolayer heart cells used in the present analysis display spontaneous activity (DeHaan, 1970) and have well-formed myofibrils (DeHaan and Hirakow, 1972). Further, a substantial proportion of the remaining nonbeating cells also have myofibrils (J. E. Rash, unpublished observations). Slightly more than 80% of the cells in heart aggregates contain myofibrils (Sachs and DeHaan, 1973) and more than 95% of the aggregates beat spontaneously (McDonald, Sachs, and DeHaan, 1972).

Monolayer tissue cultured cells had higher sodium and lower potassium concentrations immediately after trypsinization than after 1 h of suspension. During the 1 h suspension after trypsinization, cells gained about 20 mM potassium and lost about 20 mM sodium. It seems likely that trypsin digestion of the cell surface leads to an increased permeability of the membrane, and that the ion levels measured after the 1 h recovery period represent the true value. A comparable shift in sodium and potassium has been observed in HeLa cells after trypsinization and a 1 h recovery (Wickson-Ginsburg and Solomon, 1963) but was not evident in BHK cells subjected to similar treatment (McDonald, Sachs, Orr, and Ebert, 1972).

The intracellular concentrations of sodium and potassium were comparable in the intact tissue and aggregates. Lowering the extracellular potassium concentration is known to result in a loss of intracellular potassium in adult heart muscle (Gadsby et al., 1971). Similarly, when 8 day embryonic heart

was incubated in 1 mM potassium medium for 1 h, 14.1 mM sodium was gained and 11.6 mM potassium was lost in comparison to the ion levels of muscle incubated in 4.5 mM potassium. Ion levels in tissue cultured cells were affected by incubation in 1 mM potassium medium in a similar manner. The extrusion of sodium and gain of potassium during the 1 h recovery from trypsin was almost completely inhibited by incubation in low potassium medium. In addition, this recovery process was partially inhibited when the medium contained 4.5 mM potassium and 10^{-5} M ouabain suggesting that the cation pump involved in the recovery is sensitive to external potassium and to ouabain. Although 10^{-5} M ouabain had little effect on the intact heart tissue, 10^{-4} M ouabain induced a large gain of sodium and loss of potassium. Comparable changes were seen in tissue cultured cells treated with 10^{-4} M ouabain. These results are consistent with an earlier report that ouabain at concentrations less than 10^{-4} M had no significant effect on ^{42}K influx patterns in chick embryonic heart tissue (Klein and Evans, 1961). Whether this lack of sensitivity is related to development or to species will require further investigation.

The membrane potentials recorded in the intact tissue, monolayer cells, and aggregates were in good agreement. Calculated P_{Na}/P_K ranged from 0.039 in the intact tissue (7–8 days) to 0.044 in monolayer cells. Earlier reports have noted that the resting potential of tissue cultured cells was significantly lower than that of intact tissue (Lehmkuhl and Sperelakis, 1963). The results presented here probably reflect different tissue culture techniques and the use of finer-tipped microelectrodes (DeHaan and Gottlieb, 1968; Tasaki et al., 1968).

Possible Relationship of Ionic Changes to Development

The major alteration in heart electrolyte content associated with increasing embryo age was a change in the intracellular potassium concentration. From day 2 to day 14, intracellular potassium declined by 50 mM. The question arises as to whether this change in intracellular potassium reflects, for example, greater binding of cellular potassium at the younger embryo age, or whether this higher level of potassium serves a functional role in cardiac development. A large number of enzymes appear to be activated in the presence of high concentrations of potassium ions (for reviews, see Evans and Sorger, 1966; Suelter, 1970). Lubin (1967) has suggested that intracellular potassium concentration may affect the synthesis of proteins and nucleic acids. There is evidence that an increase in the intracellular potassium concentration of oocytes (*Rana pipiens*) is associated with an increase in protein synthesis (Molinaro and Hultin, 1965; Ecker and Smith, 1971). Similarly, in BHK cells in culture, there appears to be a relationship between intracellular

potassium concentration and protein synthesis (McDonald, unpublished observations). During cardiac development the heart grows rapidly from day 1 to day 3, less rapidly up to about day 8, and slowly from then on (DeHaan, 1971). Amino acid uptake in chick embryo heart decreases with increasing age (Guidotti et al., 1968). Using the mean wet weight, water content, and extracellular space of 2- to 14-day hearts, total cell wet weight was calculated, as well as the percent increase per day in total cell wet weight. Fig. 4 shows the relationship between this percentage increase and

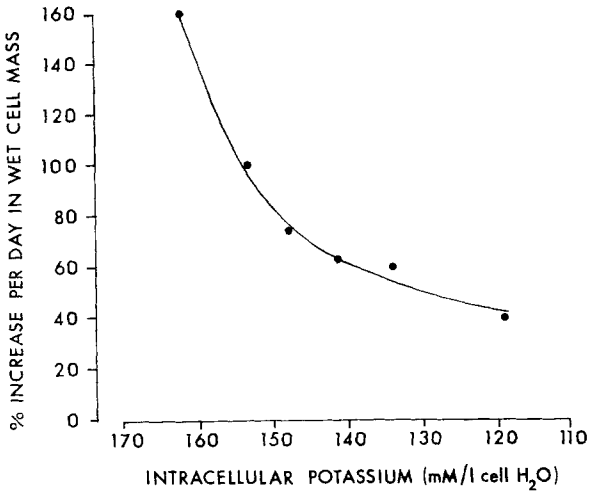

FIGURE 4. The relationship between heart growth and intracellular potassium concentration. Growth is expressed as the percent increase per day in total wet cell mass. Wet cell mass was calculated by subtracting the weight of the extracellular fluid from the mean wet weight of four hearts at each embryo age. The curve through the points was fitted by eye.

the mean intracellular potassium concentration. If, in fact, the rate of increase in total cell wet weight is a reflection of the rate of protein synthesis during that period of development, there may well be a functional relationship between protein synthesis and the intracellular concentration of potassium.

In conclusion, the data indicate that chick embryonic heart tissue develops adult-like features by days 14 to 18, in respect to its extracellular space, intracellular ion concentrations and membrane potentials. Development to this stage is characterized by declining extracellular space and intracellular potassium, an increasing resting potential, and an increasing overshoot. The membrane potentials and intracellular ion concentrations of monolayer cultured heart cells and aggregates are similar to those of intact tissue. Incubation in low potassium medium or exposure to ouabain induces comparable ion movements in cultured cells and intact tissue.

We thank Dr. H. G. Sachs for advice and assistance and acknowledge the excellent technical help of J. Wildes and E. Asch.

Received for publication 5 May 1972.

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