Changes in Membrane Properties of Chick Embryonic Hearts during Development

NICK SPERELAKIS and K. SHIGENOBU

From the Department of Physiology, University of Virginia School of Medicine, Charlottesville, Virginia 22903

ABSTRACT   The electrophysiological properties of embryonic chick hearts (ventricles) change during development; the largest changes occur between days 2 and 8. Resting potential (E_m) and peak overshoot potential ($+E_{max}$) increase, respectively, from -35 mv and $+11$ mv at day 2 to -70 mv and $+28$ mv at days 12–21. Action potential duration does not change significantly. Maximum rate of rise of the action potential ($+\dot{V}_{max}$) increases from about 20 v/sec at days 2–3 to 150 v/sec at days 18–21; $+\dot{V}_{max}$ of young cells is not greatly increased by applied hyperpolarizing current pulses. In resting E_m vs. log $[K^+]_o$ curves, the slope at high K^+ is lower in young hearts (e.g. 30 mv/decade) than the 50–60 mv/decade obtained in old hearts, but the extrapolated $[K^+]_i$ values (125–140 mM) are almost as high. Input resistance is much higher in young hearts (13 MΩ at day 2 vs. 4.5 MΩ at days 8–21), suggesting that the membrane resistivity (R_m) is higher. The ratio of permeabilities, P_{Na}/P_K, is high (about 0.2) in young hearts, due to a low P_K, and decreases during ontogeny (to about 0.05). The low K^+ conductance (g_K) in young hearts accounts for the greater incidence of hyperpolarizing afterpotentials and pacemaker potentials, the lower sensitivity (with respect to loss of excitability) to elevation of $[K^+]_o$, and the higher chronaxie. Acetylcholine does not increase g_K of young or old ventricular cells. The increase in (Na^+, K^+)-adenosine triphosphatase (ATPase) activity during development tends to compensate for the increase in g_K. $+E_{max}$ and $+\dot{V}_{max}$ are dependent on $[Na^+]_o$ in both young and old hearts. However, the Na^+ channels in young hearts (2–4 days) are slow, tetrodotoxin (TTX)-insensitive, and activated-inactivated at lower E_m. In contrast, the Na^+ channels of cells in older hearts (>8 days) are fast and TTX-sensitive, but they revert back to slow channels when placed in culture.

INTRODUCTION

The tubular heart of chick embryos begins beating spontaneously at 36–45 hr, and its contractions are coordinated by propagation of activity before the appearance of specialized conducting tissues. Desmosomes and intercalated discs

are present at day 2 (Hibbs, 1956; unpublished observations); gap junctions are not found. The myofibrils in young hearts are sparse, run in all directions, and are in various stages of formation. Sarcoplasmic reticulum is present, but a transverse tubular system is absent. Since the (Na^+, K^+)-adenosine triphosphatase (ATPase) activity of chick embryonic hearts increases during development (Klein, 1963; Sperelakis, 1972 b), the cation pumping capability of the cells is presumably enhanced. Tissue electrolyte analyses of chick embryonic hearts (ventricles) yield conflicting values for $[K^+]_i$ and $[Na^+]_i$ during development. Klein (1960) reported that $[K^+]_i$ increases from 68 mM at day 2–3 to a plateau level of 86 mM beginning at day 13. In contrast, Harsch and Green (1963) reported that the calculated $[K^+]_i$ levels actually decrease from 145 mM at day 8 to 91 mM at day 18. Total tissue Na^+ is very high in young hearts because of Na^+ binding in the extensive mucopolysaccharide cardiac jelly (Thureson-Klein and Klein, 1971), but the calculated $[Na^+]_i$ level drops to a constant level of 40 mM by day 13 (Klein, 1960). Harsch and Green (1963) reported that $[Na^+]_i$ remained constant at 23–38 mM between days 8 and 18.

Na^+ carries the inward current during the rising phase of the action potential of embryonic chick ventricular cells in intact hearts (6 and 19 days old) (Yeh and Hoffman, 1968) or in culture (Sperelakis and Lehmkuhl, 1968; Pappano and Sperelakis, 1969). Calculations from the peak overshoot potential as a function of $[Na^+]_o$ suggest that $[Na^+]_i$ is about 30 mM (Pappano and Sperelakis, 1969), and Yeh and Hoffman (1968) estimated that $[Na^+]_i$ was slightly higher in 6-day hearts than in 19-day hearts (53 vs. 47 mM). The resting and action potentials of embryonic chick ventricles as young as 6 days are large (Lehmkuhl and Sperelakis, 1963; Yeh and Hoffman, 1968). Although Fingl et al. (1952) observed no difference in their magnitudes between days 3 and 7, Shimizu and Tasaki (1966) found an increase with age. Similarly, the resting potentials of skeletal muscle increase during embryonic life in chick (Boëthius and Knutsson, 1970) and in rat, the increase in rat continuing into the early postnatal period (10–12 days) (Fudel-Osipova and Martynenko, 1964; Boëthius, 1969). The chronaxie of chick embryonic heart decreases markedly during development (Shimizu and Tasaki, 1966), and hyperpolarizing afterpotentials disappear in older hearts (Yeh and Hoffman, 1968). In addition, there is an increase in sensitivity to $[K^+]_o$ with respect to continuation of spontaneous beating (Lewis, 1929; DeHaan, 1967, 1970). We demonstrated that chick embryonic hearts are completely insensitive to tetrodotoxin (TTX) on days 2–4, are partially sensitive on days 5–7, and are completely sensitive after day 8 (Shigenobu and Sperelakis, 1971). Since increase in resting potentials, (Na^+, K^+)-ATPase activity, and sensitivity to $[K^+]_o$, decrease in chronaxie, disappearance of the hyperpolarizing afterpotential, and onset of sensitivity to TTX all suggest that changes in membrane properties occur

during development, experiments were done on embryonic chick ventricles to determine the nature of the changes. It was found that K^+ permeability increases markedly between days 2 and 7, and is responsible for many of the other observed properties. Furthermore, the Na^+ channels shift in characteristics from slow to fast.

METHODS

Fertilized chicken eggs (White Leghorn, Babcock strain) were incubated at 37°C. The embryonic hearts were removed at various stages of development from 2 days to 21 days (hatching); some experiments were done on young chicks a few days after hatching. The hearts were held in a chamber by pinning into attached surrounding tissues. For embryos 2–6 days old, because of its small size, the entire heart was usually removed and placed in the chamber; therefore, the ventricular cells were spontaneously active either by propagation of excitation from supraventricular pacemaker areas or by intrinsic automaticity. For embryos 7 days and older, usually only the ventricle was mounted in the chamber, and it was not spontaneously active. In the K^+ sensitivity experiments, the entire heart was mounted. The hearts were bathed in oxygenated Ringer solution, and maintained at 37°C (±1°C). The control Ringer solution had the following composition (in millimoles per liter): 150 Na^+, 2.7 K^+, 1.8 Ca^{++}, 1.0 Mg^{++}, 145 Cl^-, 11.9 HCO_3^-, and 1.06 $H_2PO_4^-$ (pH 7.2). Excitability and contraction continued for several hours under these conditions. The high K^+ solutions were made by substitution of KCl for NaCl in the Ringer solution, keeping the sum of $[K^+]_o + [Na^+]_o$ constant at 152.7 mM; all other ion concentrations were kept the same. The low Na^+ solutions were made by substituting choline·Cl for equimolar amounts of NaCl and using 13 mM Tris·HCl to replace the Na_2HPO_4 and $NaHCO_3$ (pH 7.4).

Most microelectrode penetrations were made into the epicardial surface, especially in the younger hearts. All impalements were made into the ventricular cells, except where indicated as atrial cells. Conventional intracellular recording was done using glass capillary microelectrodes filled with 3 M KCl. The microelectrode resistances were 30–50 MΩ. The reversible half-cells were Ag:AgCl. A W-P Instruments, Inc. (Hamden, Conn.), model M4A DC preamplifier with a high input impedance electrometer probe and negative capacitance was used, and the signal was led to a Tektronix 565 dual-beam oscilloscope (Tektronix, Inc., Beaverton, Ore.). The maximum rate of rise of the action potential and dV/dt (where V is voltage and t is time) were measured in most experiments using a Tektronix Type O operational amplifier for electronic differentiation. The input resistance (r_{in}) was measured by applying brief (e.g. 1 sec) constant-current hyperpolarizing pulses of several intensities (few nanoamperes) through the voltage-recording microelectrode using the built-in simulated Wheatstone bridge circuit of the preamplifier. The steady-state membrane potential deflection divided by the applied current (r_{in}) was then obtained by plotting the steady-state voltage/current curve and taking the slope through the origin (infinitesimally small ΔE_m). Changes in r_{in} reflect changes in membrane resistivity (R_m) if cell size and tubular geometry remain unchanged. If the resistance

between contiguous myocardial cells is relatively high (truncated cables), and the myoplasmic resistance is relatively low compared to that of the sarcolemma, then $R_m \simeq r_{in} \cdot A_s$, in which A_s is the total surface area of the cell (Sperelakis, 1969). For example, Ba^{++}, which is well known to decrease K^+ conductance, produces a large increase in r_{in} in myocardial cells (Sperelakis and Lehmkuhl, 1966; Hermsmeyer and Sperelakis, 1970). If the myocardial cells formed a simple cable, then $R_m = (r_{in}^2/R_i)$ $\pi^2 8 a^3$, in which R_i is the myoplasmic resistivity and a is the radius. If the myocardium formed a two- or three-dimensional syncytium, then r_{in} would vary with R_m raised to a power less than 0.5. Hence, regardless of the electrical arrangement of the cells, a change in r_{in} must reflect at least an equally large change in R_m, i.e. the change in R_m can be, if anything, only underestimated.

Beating or lack of beating were observed visually with a Zeiss dissecting stereomicroscope (Carl Zeiss, Inc., New York), and in some experiments contractions were recorded on a penwriter by using an AC preamplifier and a piezoelectric crystal phonograph cartridge (Astatic model 18, Astatic Corp., Conneaut, Ohio) positioned on the surface of the heart. Some hearts were driven by external electrical stimulation using platinum electrodes and brief ($\sim$5 msec) rectangular current pulses.

RESULTS

I. *Resting Potentials*

The mean transmembrane resting potential (E_m) of ventricular myocardial cells, measured by intracellular microelectrodes, increases during embryonic development, as shown in Fig. 1 and in Fig. 2 (lower curve, unfilled circles). The greatest increase is between days 2 and 7, from about -35 mv to about -63 mv, and thereafter the rate of increase is more gradual, the mean resting E_m increasing to about -70 mv at 18–24 days. Hatching occurs at 21 days. As will be discussed below, the large increase in resting E_m during the first few days may be due mainly to an increase in P_K and not in E_K. It is possible that the low recorded resting potentials in young hearts are partially exaggerated due to injury and improper sealing of the microelectrode; such current leakage around the electrode tip would be most prominent in cells having a high input resistance. However, substantially the same results were found by others, including Lehmkuhl and Sperelakis (1963), Shimizu and Tasaki (1966), and Yeh and Hoffman (1968) for embryonic chick ventricles, and by Pappano (1972) for embryonic chick atrium.

II. *Action Potentials*

As expected from the increase in resting E_m, many changes in the action potential occur during development. The peak overshoot potential ($+E_{max}$), which should be a function of the "take-off" potential (resting E_m) and of E_{Na}, increases from a mean value of about $+13$ mv at 2–3 days to $+28$ mv at 9–24 days (Fig. 1, and Fig. 2, upper curve). The amplitude of the average action potential increases from about 48 mv at day 2, to 55 mv at day 3, to

98 mv at 18–24 days, as shown by the difference between the two curves in Fig. 2. The greatest rate of increase in these two characteristics occurs early in embryonic development when the large increase in resting E_m is occurring.
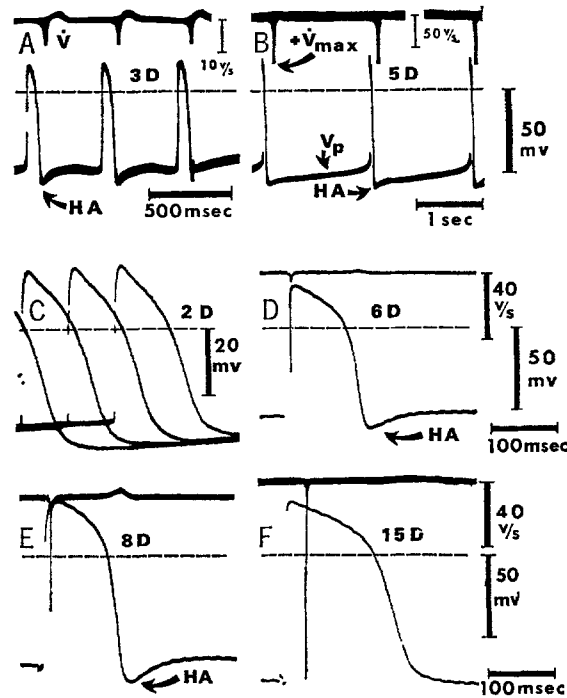

FIGURE 1.   Typical transmembrane potentials recorded from embryonic chick ventricular myocardial cells at different stages of development. *Lower traces*, action potentials; *upper traces*, dV/dt. There was a greater incidence of cells having hyperpolarizing afterpotentials and pacemaker potentials in young hearts than in older hearts. (A)–(B), slow sweep speeds to show the hyperpolarizing afterpotentials (*HA*) and pacemaker potentials (V_p) recorded from young hearts, 3 days (A) and 5 days old (B). (C)–(F), Recordings from hearts of various ages to illustrate the similarity in shape of the action potential in hearts of all ages: 2 days (C), 6 days (D), 8 days (E), and 15 days old (F). Voltage calibration is the same for all figures, except (C). Time and dV/dt calibrations are the same in (C)–(F). Action potentials in young isolated whole hearts of (A)–(C) were spontaneously generated, whereas those in older isolated ventricles in (D)–(F) were elicited by electrical stimulation. Resting potentials, action potentials, and $+ \dot{V}_{max}$ are smaller in young hearts; action potential duration and shape remain unchanged.

Substantially similar data were reported by Lehmkuhl and Sperelakis (1963), Shimizu and Tasaki (1966), Yeh and Hoffman (1968), and Nakanishi and Takeda (1969) for chick embryonic ventricular cells. Human embryonic ventricular cells (7–12 wk old) have a mean resting potential of about −85 mv, action potential of 110 mv, and overshoot of 25 mv (Tuganowski and Cekański, 1971).
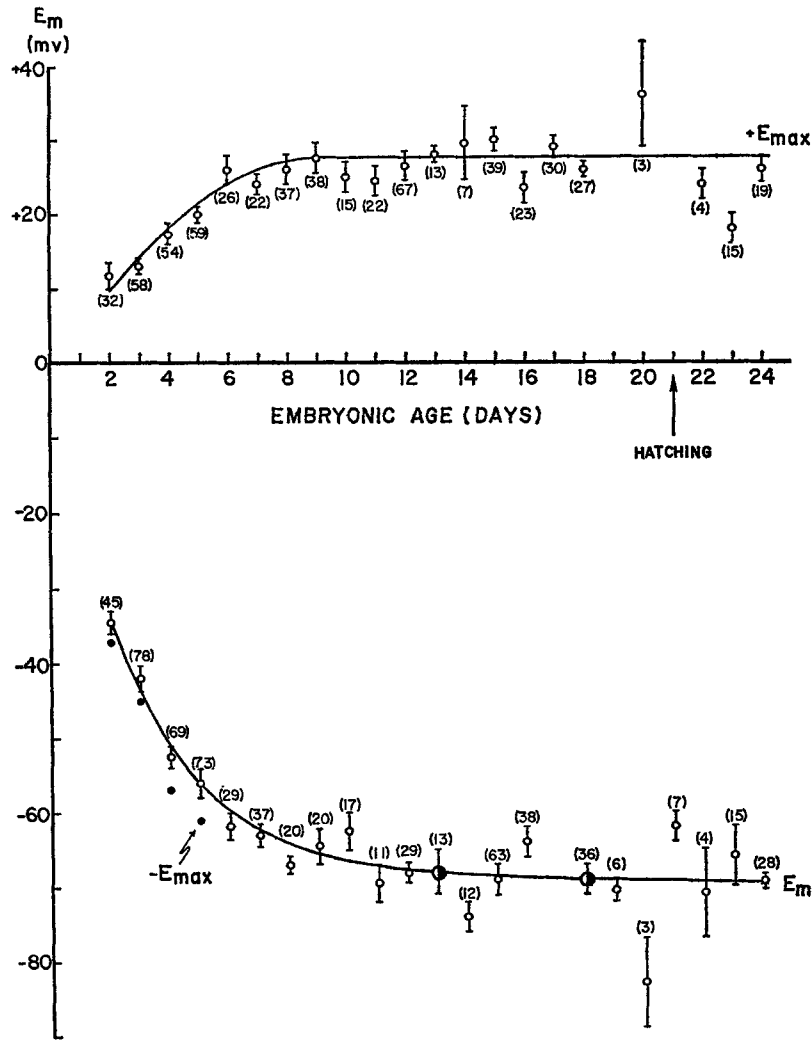
Em
(mv)
+40

+Emax

+20

(26) (22) (37) (38) (15) (22) (67) (13) (7) (39) (23) (30) (27) (3) (4) (19)
(59)
(54)
(58)
(32)

0

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

EMBRYONIC AGE (DAYS)

HATCHING

-20

(45)
(78)
(69)
(73)
(29)
-Emax
(37) (20) (20) (17) (38) (7) (15)
(11) (29) (13) (63) (36) (6) (4) (28)
(12) Em
(3)

-40

-60

-80

FIGURE 2.   Graphic representation of the resting potential (E_m), action potential peak overshoot potential ($+E_{max}$), and action potential amplitude (difference between the two curves) of intact embryonic chick hearts as a function of developmental age. These potentials increase markedly during development, the greatest changes occurring between days 2 and 8. The points plotted are the means ±1 SE. The curves were fitted by eye. The peak hyperpolarizing afterpotential or maximum diastolic potential ($-E_{max}$) are also plotted (filled circles) for some ages to show that the afterpotential, although small in young hearts, is absent in older hearts (e.g. 13 and 18 days). The estimated K^+ diffusion potential (E_K) is large in young hearts and does not increase very much during development.

The maximum rate of rise of the action potential ($+\dot{V}_{max}$) increases from a mean value of about 19 v/sec at 2–3 days, to about 80 v/sec at days 5–10, and 155 v/sec at 18–24 days (at 37°C) (Fig. 3). In agreement, Yeh and Hoffman (1968) recorded a maximum $+\dot{V}_{max}$ of 149 v/sec at day 19, but Fingl et al. (1952) reported that $+\dot{V}_{max}$ was only 14–24 v/sec between days 3 and 7. At 28°C, $+\dot{V}_{max}$ is considerably lower, as would be expected (Lehmkuhl and Sperelakis, 1963). In contrast to the action potential magnitude, the increase
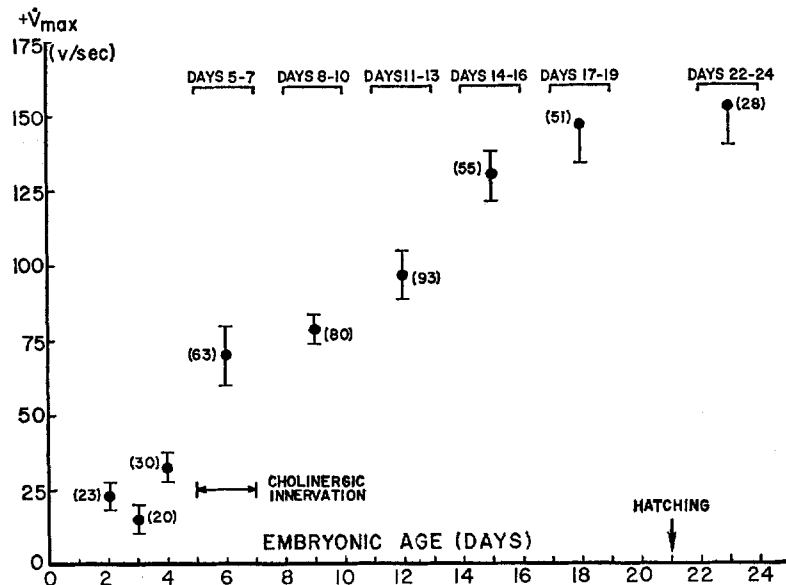

FIGURE 3. Maximum rate of rise of the action potential ($+\dot{V}_{max}$) during embryonic development. Most of the data are grouped into 3-day periods, as indicated. Although the data can be fitted by a smooth continuous curve, there may be a small jump in the data between days 4 and 6, thereby suggesting three plateau levels for $+\dot{V}_{max}$. The approximate period of cholinergic innervation of the heart is indicated. Vertical bars = ±1 SE.

in $+\dot{V}_{max}$ does not appear simply to parallel the increase in resting E_m ; instead, almost a steplike increase in $+\dot{V}_{max}$ seems to occur during a limited period encompassing days 4–6. This is about the period during which TTX-sensitive fast Na^+ channels are appearing (see below). There is a further increase in $+\dot{V}_{max}$ after day 10 (up through day 18), even though the resting E_m has reached its maximum value.

The duration of the action potential (measured at the spontaneous heart rate of about 110 beats/min in young hearts and at a driven rate of 60 beats/min in older hearts) remains essentially unchanged during development; the over-all mean for all ages was 105 msec at 50% repolarization and 130 msec at 90% repolarization. The shape of the action potential stays about the same,

and prominent plateaus are found in young as well as in old hearts. These results are essentially in agreement with those reported by Yeh and Hoffman (1968). At 26°–28°C, some hearts have very long plateaus, causing action potential durations of up to 300–600 msec (Fingl et al., 1952; Lehmkuhl and Sperelakis, 1963), as expected because of the effect of cooling on the kinetics of the ion channels and on the frequency of spontaneous firing.

III. *Afterpotentials and Pacemaker Potentials*

The occurrence of hyperpolarizing (positive) afterpotentials (HA) and of pacemaker potentials (V_p) (slow diastolic depolarization) in the young ventricular cells is quite variable, but there was a greater tendency of the younger cells

TABLE I

THE INCIDENCE OF HYPERPOLARIZING AFTERPOTENTIALS
DURING EMBRYONIC DEVELOPMENT OF
CHICK HEARTS (VENTRICLES)

Embryonic age	N	Amount of cells exhibiting hyperpolarizing afterpotentials*
days		%
2–3	90	81
4–5	76	63
6–8	34	38
9–21	46	0

N, total number of impalements counted.
* The remaining fraction of the cells either did not exhibit any afterpotential or had a depolarizing afterpotential.

to demonstrate these characteristics than the older cells (Table I). In young hearts, most impaled cells exhibited hyperpolarizing afterpotentials or pacemaker potentials. However, some cells impaled in hearts between days 2 and 5 did not have either component. In agreement, Yeh and Hoffman (1968) reported that the hyperpolarizing afterpotentials (6–10 mv) characteristic of 6-day old chick ventricles disappeared in 19-day old hearts. The presence of large hyperpolarizing afterpotentials in young hearts (i.e. $-E_{max} > E_m$) is consistent with the finding (discussed below) that E_K is considerably greater than the resting E_m, and the presence of pacemaker potentials is consistent with a low steady-state P_K.

IV. *Resting Potential vs. $[K^+]_o$*

The relationship between resting potential and log $[K^+]_o$ is illustrated in Fig. 4 for three representative hearts. A total of 21 hearts were analyzed and linear curves were fit mathematically (method of least squares) to the data points above 10 mM $[K^+]_o$, and the slopes and extrapolated $[K^+]_i$ values were ob-

tained from these fitted curves (Table II). The data for older embryonic hearts is like that for adult hearts: linear at $[K^+]_o$ levels above 10 mM with a slope
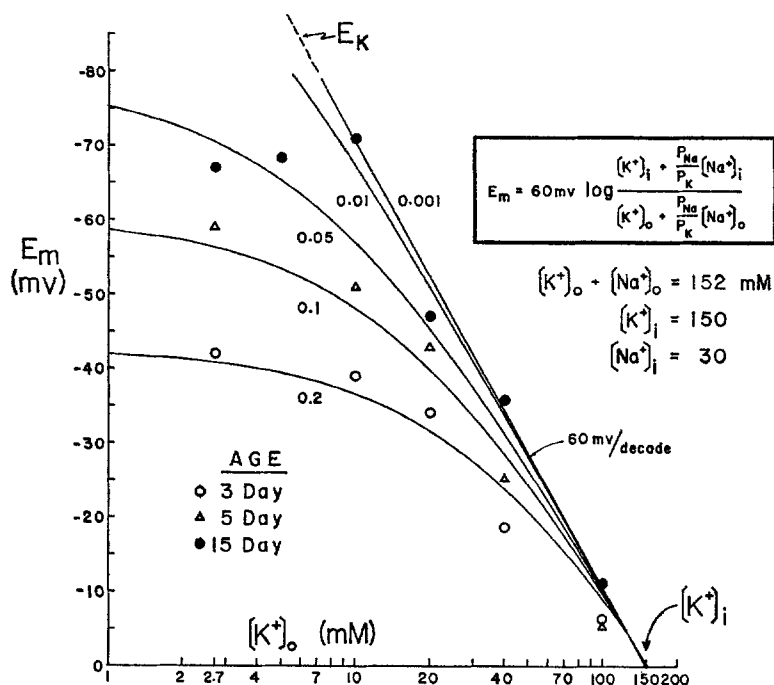

FIGURE 4.   Resting potential (E_m) plotted as a function of $[K^+]_o$ on a logarithmic scale. $[K^+]_o$ was elevated by substitution of K^+ for equimolar amounts of Na^+. Continuous lines give theoretical calculations from the constant-field equation (shown in *inset*) of resting E_m as a function of $[K^+]_o$ for various assumed P_{Na}/P_K ratios of 0.001, 0.01, 0.05, 0.1, and 0.2. Calculations were made assuming a $[K^+]_i$ of 150 mM, $[Na^+]_i$ of 30 mM (estimated from $[Na^+]_o$ level at which excitability is lost); the sum of $[K^+]_o$ + $[Na^+]_o$ was held constant at 152 mM, which was the method used to obtain the experimental data given here and in Table II. As indicated by the equation, the shapes of these theoretical curves are dependent on the assumed values for $[Na^+]_i$. For a P_{Na}/P_K ratio of 0.001, the curve is linear over the entire range with a slope of 60 mv/decade, i.e. it closely follows E_K ; at higher ratios, the slope continually diminishes as $[K^+]_o$ is lowered, and the curve flattens at low $[K^+]_o$ levels. Symbols give representative data obtained from embryonic chick hearts at days 3, 5, and 15. The data points for the 3-day heart follow the curve for a P_{Na}/P_K ratio of 0.2, those for the 5 day heart follow the theoretical curve for 0.1, and those for the 15 day heart follow the curve for a ratio of 0.01. The estimated intracellular K^+ activity $([K^+]_i)$ levels obtained by extrapolation to zero potential are nearly the same for hearts of all ages.

approaching the theoretical 60 mv/decade, indicating a virtually completely K^+ selective membrane in high $[K^+]_o$. Thus, the resting E_m is nearly equal to E_K at high $[K^+]_o$ but deviates from E_K at lower $[K^+]_o$ levels. The decrease in E_m in some of the older hearts as $[K^+]_o$ is lowered from 10 to 2.7 mM could be

due to a dependence of P_K on $[K^+]_o$. In younger hearts, besides beginning at a lower resting potential for the control $[K^+]_o$ of 2.7 mM, the average slope at high $[K^+]_o$ levels (between 10 or 20 and 100 mM) is considerably less (Fig. 4; Table II). The slope is about 30 mv/decade for a 2 day old heart, 40 for 3-day hearts, 45 for 4-day hearts, and generally 50–60 mv/decade for older hearts. Also, at lower $[K^+]_o$ levels, the curve is flatter. It must be noted, however, that if the recorded potentials at all $[K^+]_o$ levels for the young hearts

TABLE II

SUMMARY OF THE DATA OBTAINED FROM E_m VS. LOG $[K^+]_o$ CURVES FOR
CHICK EMBRYONIC HEARTS (VENTRICLES) OF VARIOUS AGES

Embryonic age	N	Average E_m (at $[K^+]_o$ = 2.7 mM)	Slope	Extrapolated $[K^+]_i$*	Average P_{Na}/P_K ratio
days		*mv*	*mv/decade*	*mM*	
2	1	−40	−29	125	0.21
3	2	−45	−40	130	0.17
4	1	−57	−45	140	0.08
4	3	—	−46‡	145‡	—
5	3	−55	−50	130	0.08
6	3	—	−50‡	125‡	—
7–9	5	−65	−50	145	0.07
11–12	4	−67	−52	145	0.07
12	3	—	−53‡	145‡	—
14–18	5	−68	−51	155	0.05
18	2	—	−59‡	125‡	—

N is the number of hearts.
The slope is the average at $[K^+]_o$ levels of 10 mM and higher.
The P_{Na}/P_K ratio was calculated from the constant-field equation at every $[K^+]_o$ level for which E_m was measured and the average value was calculated for each heart; some individual values for hearts in the 14–18 day group were as low as 0.025.
* $[K^+]_i$ was estimated to the nearest 5 mM from the intersection of fitted linear curves with abscissae.
‡ These data were taken from Pappano (1972) for embryonic chick atrium.

were a constant fraction of the true potential due to improper electrode sealing, then the slope of the curve would be underestimated. Estimates of the intracellular K^+ activity ($[K^+]_i$) obtained by extrapolation of the linear portion of the fitted curves to zero potential indicates that $[K^+]_i$ is quite high in young hearts, e.g. it is already about 120 mM in 2-day old myocardial cells, and that the $[K^+]_i$ level does not greatly increase during development (Fig. 4; Table II). The values of $[K^+]_i$ for all hearts studied (all ages) fell between 110 and 180 mM, but there is a tendency for the younger hearts to have the lower levels. Values of 140–180 mM are typical for adult myocardial cells. Pappano (1972) found for the atrial cells of embryonic chick hearts that the slope between 40 and 100 mM $[K^+]_o$ also was lower for young cells, being only 46 ± 2

mv/decade at day 4 and 50 ± 2 at day 6 (Table II); the extrapolated $[K^+]_i$ values were also fairly constant, and ranged only between 120 and 150 mM.

The findings in young hearts of considerably lower resting potentials and slopes of the E_m vs. log $[K^+]_o$ curves, combined with a relatively high $[K^+]_i$, suggests that the ratio of Na$^+$ permeability (P_{Na}) to K$^+$ permeability (P_K) is much higher than in older hearts. Theoretical curves calculated from the constant-field equation are plotted in Fig. 4 for different ratios of P_{Na}/P_K (assuming P_{Ca} is negligibly small). For a P_{Na}/P_K ratio of 0.001, the calculated curve is nearly linear over the entire range with a slope of 60 mv/decade, i.e. it closely follows E_K, as calculated from the Nernst equation. At higher P_{Na}/P_K ratios, the slope continually diminishes as $[K^+]_o$ is lowered, and the curve becomes flat; the resting E_m at physiological $[K^+]_o$ levels is considerably smaller. Note the similarity of the E_m values predicted from the theoretical curves for P_{Na}/P_K ratios of 0.2, 0.1, and 0.01, respectively, with the experimental data obtained from 3-day, 5-day, and 15-day old hearts. P_{Na}/P_K ratios of 0.01–0.05 are typical for many adult nerve and muscle membranes. Thus, it appears that P_{Na}/P_K is high in young embryonic hearts and rapidly approaches the adult value within 1 wk. Since the magnitude of P_{Ca} relative to P_{Na} is not known, it might be more accurate to say that P_{Na}/P_K and/or P_{Ca}/P_K is higher in the young hearts.

V. *Input Resistance vs. Age*

The input resistance (r_{in}) of embryonic myocardial cells is high in young hearts and rapidly declines to the final adult value before day 8 (Fig. 5). The average r_{in} is 13 ± 1.5 MΩ at day 2 compared to 6.7 ± 0.7 MΩ at day 4, and 4.5 MΩ at days 11–24. Assuming that the average cell size and the electrical arrangement of the cells remains unchanged during this period, membrane resistivity (R_m) should be higher $(g_K$ lower) in the younger hearts (since there is no T-tubular system in chick myocardial cells) (see Methods). This suggests that the ratio of P_{Na}/P_K is high in young hearts because P_K is low and not because P_{Na} (and/or P_{Ca}) is high. The steady-state voltage/current curves, from which r_{in} was obtained, were nearly linear for most cells up to 20 mv hyperpolarization; a few cells showed anomalous rectification, R_m decreasing with large hyperpolarizations. In young hearts, cells having a lower resting E_m tend to have a higher r_{in}, as expected. It should be noted that, if there was improper electrode sealing in the case of the young hearts, r_{in} would be underestimated.

VI. *Sensitivity to* $[K^+]_o$

Since flattening at lower $[K^+]_o$ levels is more prominent for young hearts, i.e. the younger hearts depolarized somewhat less by a given increment in $[K^+]_o$ (see Fig. 4), experiments were done to test whether the critical $[K^+]_o$ level at

which elicited membrane excitability and contractions are abolished is higher
in younger hearts. The ventricles of young hearts appear to tolerate a slightly
higher $[K^+]_o$ level before failure occurs (Fig. 6, unfilled circles). In younger
hearts (2–5 days old), failure occurs at about 25 mM, whereas in older hearts
(15 days and older) loss of excitability occurs at about 20 mM $[K^+]_o$. Super-
imposed in Fig. 6 are previously published data (Sperelakis et al., 1970) on
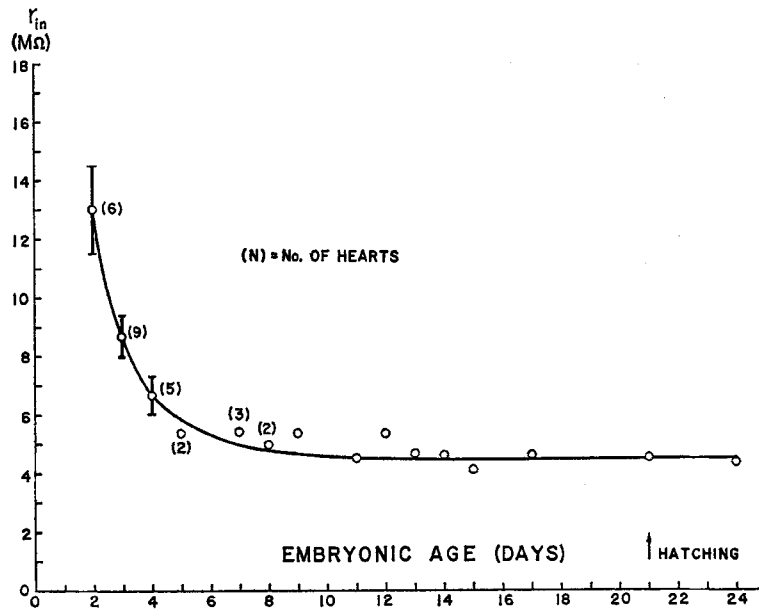failure of excitability in old embryonic chick hearts and adult cat heart. This

FIGURE 5. Input resistance (r_{in}) of intact chick ventricular cells as a function of em-
bryonic age. r_{in}, and therefore probably membrane resistivity, decreases markedly
between day 2 and day 8. The numbers in parentheses give the number of hearts sampled
at each age; only one heart was sampled for those data points without numbers. The
means and standard errors were calculated with respect to the number of hearts sampled.
The number of penetrations and measurements averaged to obtain the mean for each
heart was 4–6 for the younger hearts and 10–20 for the older hearts.

result can be explained by the fact that the percentage depolarization is only
slightly greater in young hearts; that is, for inactivation of the Na^+ channels,
the relative degree of depolarization from the normal resting potential, and
not the absolute, may be the main determining factor. However, with respect
to automaticity of the nodal cells, the young hearts can tolerate a considerably
higher $[K^+]_o$ level before automaticity is completely depressed (Fig. 6, filled
circles). In all hearts the spontaneous frequency of beating diminished as
$[K^+]_o$ was elevated above 10–15 mM (frequency actually increased in some
hearts with elevation up to 15 mM). Complete cessation of spontaneous activity

occurred at about 25 mM K$^+$ in young hearts (2–5 days old) compared to about 15 mM for old hearts (15 days old). Thus, with respect to the electrogenesis of pacemaker potentials in nodal cells, there must be a distinct difference as a function of age.
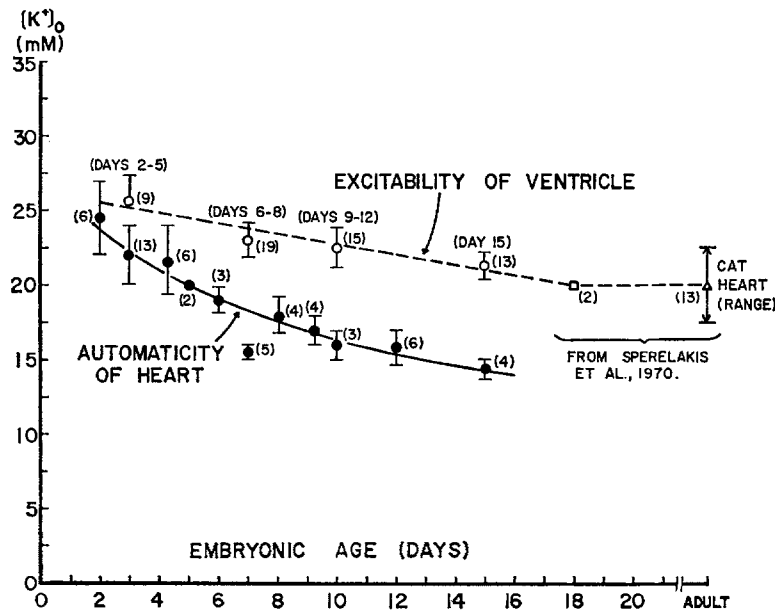

FIGURE 6. Data summarizing the sensitivity to [K$^+$]$_o$ of chick hearts at various stages of development. The ordinate gives the critical [K$^+$]$_o$ level at which (a) membrane excitability and contractions of the ventricular myocardium in response to electrical stimulation are abolished, or (b) automaticity of the heart completely disappears. [K$^+$]$_o$ was increased in increments of 2.5 mM. The bath was usually changed for each increment (KCl substituted for NaCl), but in a few experiments the bath was changed once (to 10 or 15 mM [K$^+$]$_o$) and thereafter small portions of concentrated KCl were added to elevate [K$^+$]$_o$ further (slightly hypertonic, but smaller decrease in [Na$^+$]$_o$). There were no differences in results of these two methods. The ventricular myocardial cells in young hearts are only slightly less sensitive to [K$^+$]$_o$, with respect to abolition of excitability, but the nodal pacemaker cells are considerably less sensitive, with respect to cessation of automaticity.

The young hearts are nearly completely insensitive to acetylcholine (ACh$^+$) (10^{-6}–10^{-4} M), even though a large hyperpolarization is theoretically possible because E_K is much greater than the resting E_m. ACh did not decrease the action potential duration. Therefore, it is likely that ACh$^+$ does not significantly increase P_K in ventricular cells. There was a transient (1–2 min) decrease in frequency of spontaneous firing, which may have been due to an action on the atrial and nodal pacemaker areas left attached in the case of the young hearts or on the pacemaker potentials that some of the young ven-

tricular cells exhibited. In older embryonic ventricles driven at a constant rate, ACh+ also had essentially no effect; in contrast, on the atrial action potential, ACh produced a pronounced, rapid, and persisting decrease in duration. Thus, both young and old ventricular cells do not appear to possess ACh+ receptors. These results are in agreement with Nakanishi and Takeda (1969), Coraboeuf et al. (1970), and Pappano (1972).

VII. *Characterization of the Channels which Pass the Inward Current during the Action Potential*

(A) $+\dot{V}_{max}$ vs. E_m The effect on $+\dot{V}_{max}$ of variations in resting E_m, accomplished by applying polarizing current pulses of long duration, was examined for hearts of various ages. It was found that the $+\dot{V}_{max}$ of myocardial cells in older embryonic hearts is affected by changes in the "take-off" E_m as in adult hearts. Namely, hyperpolarization causes a small or no increase in $+\dot{V}_{max}$, whereas depolarization produces a large decrease (Fig. 7). This effect is usually explained by progressive fast Na+ channel inactivation with greater depolarizations, and zero inactivation when the membrane is sufficiently hyperpolarized. Thus, these data suggest that the Hodgkin-Huxley h factor is large at the normal resting E_m. Since at a steady-state E_m of -50 to -60 mv all of the fast Na+ system should be inactivated, this makes it further unlikely that the low $+\dot{V}_{max}$ obtained in young 2- to 3-day old hearts is due to a partially inactivated fast Na+ channel system. In young myocardial cells also, although small depolarization produced pronounced decrease in $+\dot{V}_{max}$, large hyperpolarizations only slightly increased $+\dot{V}_{max}$ (Fig. 7). That is, although the normal resting E_m is low in young hearts, hyperpolarizing a cell to levels comparable to the normal resting E_m of older cells does not cause comparably large $+\dot{V}_{max}$ values (see Fig. 3). Therefore, the low $+\dot{V}_{max}$ and slow Na+ channel characteristics of young hearts are not due to inactivation of a fast Na+ channel system because of the low resting E_m levels; instead, the fast Na+ channel system seems to be absent in young hearts. Fig. 7 shows that complete inactivation of the 13 day heart occurs at -58 mv, whereas it doesn't occur until about -25 mv in the 3-day and 5-day hearts. Although it is not possible to distinguish from the present data, it is possible that the 5-day hearts in the transition period have two sets of channels, one set inactivating at about -50 mv and the other set at -25 mv. Attempts at producing hyperpolarization of all the cells in young hearts by alternative methods, adding Sr++ (1–5 mM) (Sperelakis and Lehmkuhl, 1966) or ACh+ (see above), failed.

(B) effect of variation in $[Na^+]_o$ on the action potential Loss of excitability in Na+-free solution (bath changed several times in rapid succession) occurred in both young and old embryonic chick hearts, in confirmation of our recent report for young hearts (Shigenobu and Sperelakis, 1971). The spontaneous and/or elicited electrical and mechanical activities usually disap-

peared within several minutes in old hearts and within 10–30 min in young hearts (which contain mucopolysaccharide cardiac jelly known to bind Na^+ [Thureson-Klein and Klein, 1971]). The young and old myocardia failed to respond to electrical stimulation of 10-fold greater current intensity than normal, and excitability returned within a few minutes after reintroduction of
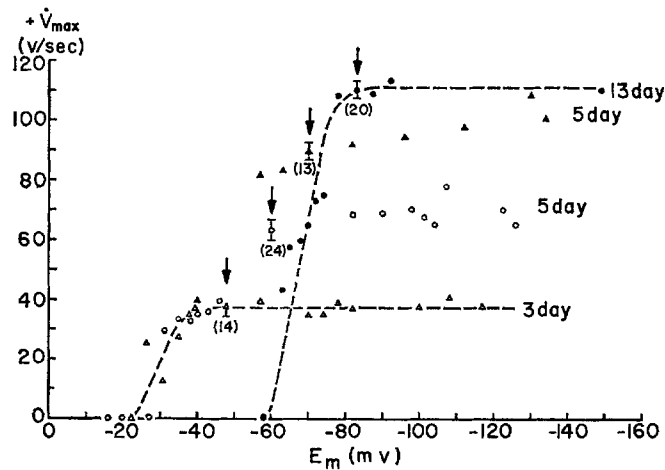

FIGURE 7. Data illustrating changes in maximum rate of rise of action potential (+ $\dot{V}_{max}$) as a function of E_m for four representative hearts of different embryonic ages (3, 5, 5, and 13 days). E_m was changed by applying rectangular polarizing current pulses of long duration (several seconds). Each type of symbol represents measurements on the same cell in one heart and each point is the average of two to five repetitions (not done consecutively). The arrows point to the mean (±1 SE, N = number in parentheses) obtained at the natural resting potential (polarizing pulses not applied). Large hyper-polarizations only slightly increased + $\dot{V}_{max}$ even in young hearts in which the normal resting E_m is very low, thus ruling out preponderant channel inactivation. Note that complete inactivation for the 13 day heart occurs at about −58 mv, whereas complete inactivation doesn't occur until about −25 mv for the 3-day and 5-day hearts. The broken curves were fitted by eye to the data for the 3-day and 13-day hearts, i.e., those hearts in which there appears to be only one type of Na^+ channel: slow and fast, respectively. The data for the 5-day hearts (transition period) suggest the possibility that there are two sets of channels, one set inactivating at about −50 mv and the other set at −25 mv.

Na^+. The peak overshoot potential ($+E_{max}$) and the maximum rate of rise of the action potential ($+\dot{V}_{max}$) were dependent on $[Na^+]_o$, as illustrated in Fig. 8. The quantitative relationships between $+E_{max}$ and $+\dot{V}_{max}$ as a function of $[Na^+]_o$ are given in Fig. 9 for hearts of various embryonic ages. At lower levels of $[Na^+]_o$, the $+E_{max}$ curves are nearly linear with slopes approaching 60 mv/decade, indicating that the major inward current during the action potential of both young and old hearts is carried by Na^+. Failure of excitability usually occurs at a $[Na^+]_o$ level of 25–30 mM. Yeh and Hoffman (1968) had

previously reported a slope of about 60 mv/decade for $+E_{max}$ vs. log $[Na^+]_o$ in both 6-day and 19-day old embryonic chick ventricular cells, and loss of excitability when $[Na^+]_o$ was reduced to 20% (31 mM) using sucrose replace-
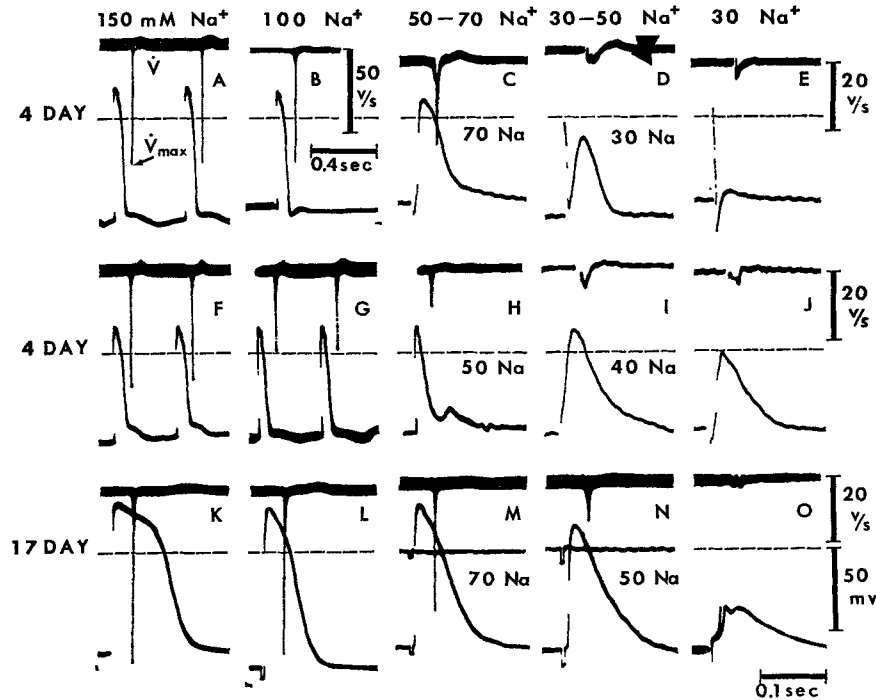

FIGURE 8.   Representative recordings illustrating the effect of lowering of $[Na^+]_o$ on $+\dot{V}_{max}$ and E_{max} of cells in young (4 day old, A–E and F–J) and old (17 day old, K–O) embryonic chick ventricles. The upper trace in each panel gives dV/dt, and $+\dot{V}_{max}$ is the peak deflection of this trace. *First column:* control records obtained in 150 mM $[Na^+]_o$; *other columns:* records at lower $[Na^+]_o$ as indicated (exact $[Na^+]_o$ given in each panel for third and fourth columns). Calibration for $+\dot{V}_{max}$ given at the right side of each row applies to that row, with the exception that the calibration given in (B) applies to (A)–(B); the V calibration given in (O) applies to all panels. The time calibration given in (B) applies to (A), (B), (F), (G), and (H), and that given in (O) applies to the remainder. Action potentials were elicited by electrical stimulation, except those in (A), (B), (F), (G), and (H) which were spontaneous. The middle horizontal trace in (M) and (N) represents the zero potential level after pull-out immediately after the recorded responses; the broken lines in all other panels give the zero potential level. At least 15 min was allowed for equilibration in each solution.

ment. The $+\dot{V}_{max}$ curves for the young hearts (3- and 4-day old) are linear over the entire range of $[Na^+]_o$ levels. For the old heart (17 day old), the curve is nearly linear at the lower levels of $[Na^+]_o$, and flattens at the higher levels. These data indicate that the major inward current carrier is Na^+ in both young and old hearts. Since the Ca^{++} concentration in the Na^+-free and low
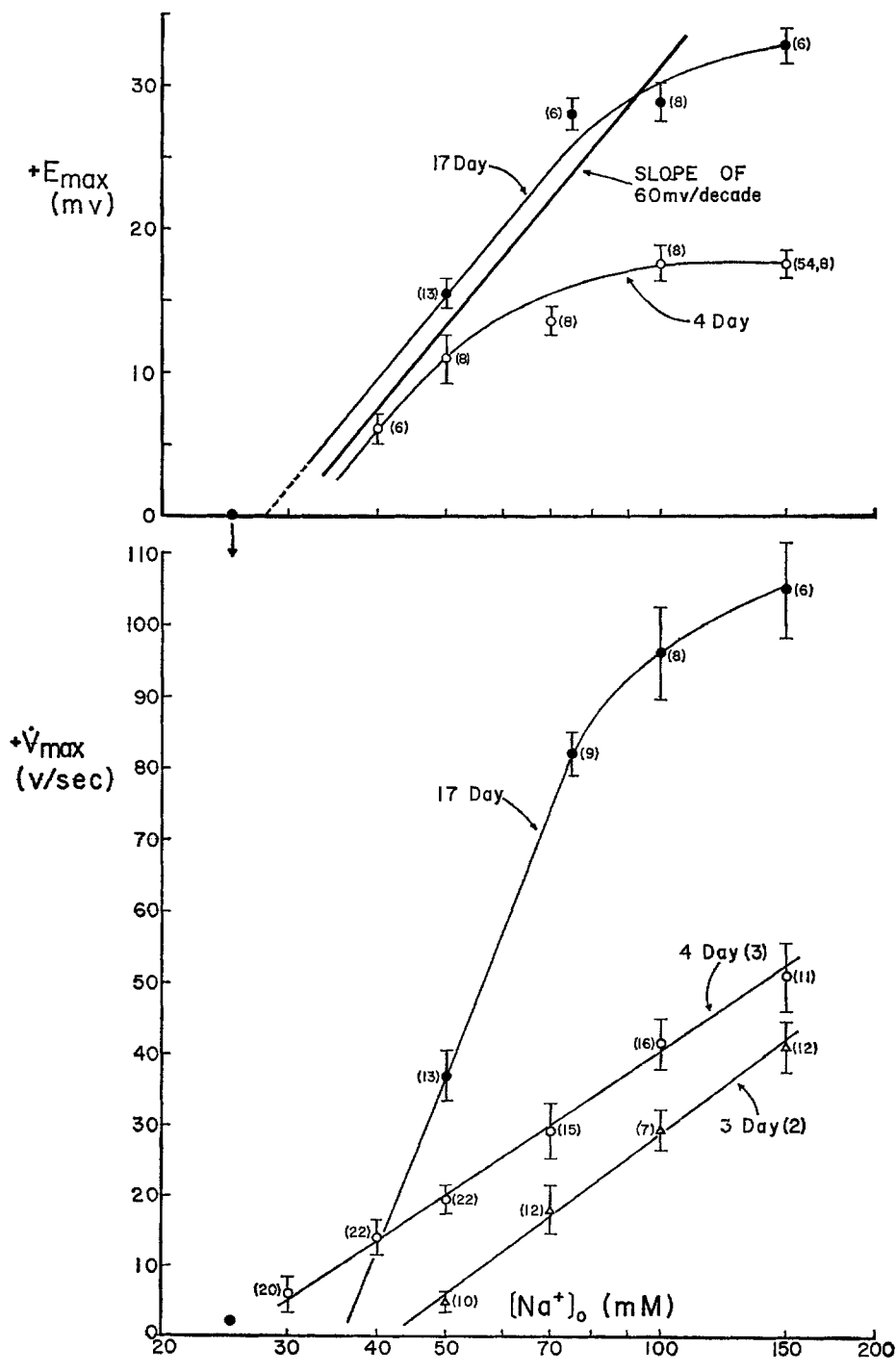
Na^+ solutions was unchanged, it is unlikely that a large inward Ca^{++} current flows during the action potential. Vereecke and Carmeliet (1971) also found the theoretically predicted linear relationship between $+ \dot{V}_{max}$ and log $[Sr^{++}]_o$ at the lower $[Sr^{++}]_o$ levels (whose slope is a function of the activation and inactivation factors for $\bar{g}_{Sr}$ and C_m) for Sr^{++} action potentials in sheep Purkinje fibers.

(c) SENSITIVITY TO TTX   In confirmation of our previous report (Shigenobu and Sperelakis, 1971), TTX has no effect on the action potentials of young embryonic hearts 2–3 days old, but usually is completely effective (i.e. $+ \dot{V}_{max}$ reduced to zero) in hearts older than 7 days (Table III). In hearts 5–6 days old, TTX usually has a partial effect, i.e. $+ \dot{V}_{max}$ is reduced, but not to zero. It appears that, regardless of exact age, cells having a high $+ \dot{V}_{max}$ (e.g. >65 v/sec) are completely sensitive, cells having a low $+ \dot{V}_{max}$ (<20 v/sec) are completely insensitive, and cells having an intermediate $+ \dot{V}_{max}$ (20–60 v/sec) are partially sensitive to TTX.

DISCUSSION

The results indicate that several membrane electrical properties are different in young chick embryonic ventricular myocardial cells. For one, P_K is low and it rapidly increases, reaching its final adult value by day 10. This can account for many of the electrophysiological properties of the young cells, including the low resting potential. The low resting potential in turn can account for some things, such as the small amplitude of the action potential. Although the low $+ \dot{V}_{max}$ in young cells is consistent with partial inactivation of fast Na^+ channels because of the low resting potential, the TTX insensitivity suggests that the young cells have a second important difference, namely the presence of a slow Na^+ channel system rather than a fast channel. This can account for many other of the electrophysiological properties of the young cells, including their insensitivity to TTX, their low $+ \dot{V}_{max}$ which isn't greatly

FIGURE 9.   Evidence suggesting that the major inward current is carried by Na^+ in both young and old hearts. The effect of variation in $[Na^+]_o$ on the peak overshoot potential $(+E_{max})$ (upper graph) and on maximum rate of rise of the action potential $(+\dot{V}_{max})$ (lower graph) is illustrated. $[Na^+]_o$ (plotted on a logarithmic scale) was lowered by replacing the NaCl with equimolar amounts of choline·Cl. The total number of impalements are given in parentheses. *Upper graph*, plotted are the mean $+E_{max}$ values (± 1 SE bars) of multiple penetrations in one 4 day old heart and one 17 day old heart. A heavy line having a slope of 60 mv/decade has been arbitrarily superimposed. The curves are nearly linear with a slope approaching 60 mv/decade at lower $[Na^+]_o$, but flatten at higher $[Na^+]_o$ levels. *Lower graph*, plotted are the mean $+\dot{V}_{max}$ (± 1 SE) values of multiple penetrations into two 3 day old hearts, three 4 day old hearts, and one 17 day old heart. The curve for the old heart is linear at lower $[Na^+]_o$ levels, and flattens at the higher levels; for the young hearts, the curves are linear over the entire range.

increased by applied hyperpolarizing current pulses, and their continued ability to fire action potentials at low resting potentials. A third difference is that the specific activity of the membrane (Na^+, K^+)-ATPase is low in young hearts and increases during development (Klein, 1963; Sperelakis, 1972 b). Therefore, the activity of the cation pump is probably lower in young hearts. Since $[K^+]_i$ is quite high and $[Na^+]_i$ is relatively low in young cells, the low $Na^+:K^+$ pump activity must be sufficient to maintain this large E_K and E_{Na} .

TABLE III

EFFECT OF TETRODOTOXIN (TTX) ON THE MAXIMUM RATE
OF RISE OF THE ACTION POTENTIAL $(+\dot{V}_{max})$ OF CHICK
HEARTS (VENTRICULAR MUSCLE)

Embryonic age	N	Control + $\dot{V}_{max}$	Effect of TTX*
days		*v/sec*	
2-3	2‡	<20	None
5-6	2	<20	None
	5	20-60	Partial
	2	20-60	Complete
7-8	1	<20	None
	5	20-60	Complete
	1	>65	Complete
9-10	2	20-60	Complete
	4	>65	Complete
11-13	2	20-60	Partial
	3	20-60	Complete
	8	>65	Complete
14-16	8	20-60	Complete
	41	>65	Complete
17-19	8	>65	Complete

* Partial effect: reduction of $+\dot{V}_{max}$ to <20 v/sec. Complete effect: reduction of $+\dot{V}_{max}$ to 0.
‡ Many other young hearts (2–4 days) were examined, and exhibited continued spontaneous activity in TTX, but control $+\dot{V}_{max}$ values were not recorded.

This may be made possible by the low g_K because of its depolarizing action; this causes a lower electrochemical driving force for inward Na^+ current and hence a lower Na^+ leak. Thus, the pump rate need only be low to maintain a high ratio of $[K^+]_i/[Na^+]_i$ when the K^+ permeability is low. As P_K increases during development, so does the activity of the (Na^+, K^+)-ATPase, although the latter continues after P_K has leveled off (Sperelakis, 1972 a, b). Thus, the increase in resting potential during development is probably due to two factors: (a) increase in P_K, thereby decreasing the P_{Na}/P_K ratio, and (b) increase in activity of the $Na^+:K^+$ pump, thereby increasing E_K somewhat. The first factor is mainly responsible for the large increase in resting E_m between days 2 and 8 and the second factor may be primarily responsible for

the slow increase in E_m after day 9. The present results suggest that there may be a small increase in $[K^+]_i$ during development. Maintenance of a high $[K^+]_i$ may be desirable for the optimal operation of a variety of enzymes involved in cellular metabolism, protein synthesis, and growth. The energy the cell need expend for pumping cations would be less in the younger hearts because of the smaller cation leak.

The increase in P_K could reflect an increase in the number of K^+ channels per unit surface area of membrane and/or in the conductance per K^+ channel. If the former, the density of *functional* K^+ channels in resting membrane may be lower in younger cells. The estimated surface density of (Na^+, K^+)-ATPase molecules is several orders of magnitude greater than that for resting K^+ channels. It is difficult to be certain that the low P_K is the cause of the low resting E_m, rather than the other way around, i.e., a low E_m causes the low P_K due to anomalous rectification (diminution of P_K with partial depolarization). However, P_K may be altered by changes in E_m with applied polarizing current pulses. A given myocardial cell seems to change gradually and doesn't shift suddenly from the low P_K to the high P_K state. Much of the P_K increase during development probably precedes the arrival of the cholinergic innervation. P_K is increasing between days 2 and 4, whereas the parasympathetic nerves just begin to penetrate into the heart during the 5th day, with innervation being nearly completed by day 7 (Szepsenwol and Bron, 1936; Romanoff, 1960). However, one might expect some variation in the precise period of innervation, depending on many possible factors, such as variation from one embryo to another, incubation temperature, etc. Adrenergic neurons do not appear until about the 16th day (Enemar et al., 1965).

The low P_K of young myocardial cells accounts for the high membrane resistivity (R_m) suggested by the present results, as well as for the high chronaxie (σ) in young chick embryonic hearts (Shimizu and Tasaki, 1966). There is a decrease in σ from 4 msec at day 2 to about 2 msec at day 5, and to 1 msec at days 11–16; most of the change occurs by day 8. If C_m remains constant, the high chronaxie found in young hearts suggests that R_m is greater in agreement with the input resistance measurements made in the present study. However, the relationship between chronaxie and time constant in cardiac muscle may be complicated (e.g., see Dominguez and Fozzard, 1970). It is interesting that, from measurements of K^+ efflux and Na^+ influx, P_K increases approximately fourfold whereas P_{Na} remains relatively constant in Asterias embryos shortly after fertilization, and the decrease in P_{Na}/P_K can account for the increase in resting potential (Tupper and Powers, 1972).

The low P_K in young myocardial cells also accounts for the observed high incidence of hyperpolarizing afterpotentials, automaticity, and lower sensitivity to elevated $[K^+]_o$. Yeh and Hoffman (1968) found disappearance of the hyperpolarizing afterpotential between day 6 and day 19, and they concluded

from this that g_K increases during development by inferring that resting E_m departs further from E_K in younger hearts than in older hearts. The low P_K in young ventricular cells would be expected to produce a greater degree of automaticity (for a given low P_{Cl}/P_K ratio) (Trautwein, 1963; Sperelakis and Lehmkuhl, 1966; Sperelakis et al., 1967). Lewis (1929) demonstrated that 3-day old embryonic chick hearts continue to beat, although at a reduced rate, in Ringer solutions containing 17 mM $[K^+]_o$, whereas older hearts had stopped beating. DeHaan (1967, 1970) reported that, between days 2 and 7, there was a gradual increase in sensitivity to $[K^+]_o$, with respect to the percentage of isolated single cells in culture which continued to beat spontaneously, and he inferred from this that P_K was low in young cells.

Young hearts between days 2 and 4 (low $+\dot{V}_{max}$) are not sensitive to tetrodotoxin (TTX), whereas hearts 8 days old or older (large $+\dot{V}_{max}$) are sensitive (Shigenobu and Sperelakis, 1971). In old hearts, $+\dot{V}_{max}$ and $+E_{max}$ progressively diminish until excitability fails. On days 5 and 6, some hearts are sensitive to TTX whereas others are not: cells having a low $+\dot{V}_{max}$ are not sensitive, whereas those with 65 v/sec or more are completely sensitive (i.e. $+\dot{V}_{max}$ goes to zero) (see Table III). Hearts having an intermediate $+\dot{V}_{max}$ (e.g. 20–60 v/sec) are partially sensitive ($+\dot{V}_{max}$ is reduced). Thus, it appears that only TTX-insensitive slow Na^+ channels are present in embryonic hearts between days 2 and 4, that mainly TTX-sensitive fast Na^+ channels are present in hearts 8 days old or older, and that there is a transition period between days 5 and 6 when a given cell may have both slow and fast Na^+ channel systems simultaneously. However, we do not wish to imply a rigid time-course to these changes because of the possibility of a great deal of variability from one embryo to another, as indicated in Table III. Renaud and Le Douarin (1972) observed that sensitivity to TTX (0.5 μg/ml) and to Mn^{++} (2 mM) in the chick ventricle was present at about the 40 somite stage, which corresponds to the beginning of the 5th day of development if the incubation temperature is 36°C (Romanoff, 1960). Ishima (1968) has previously shown that chick embryonic hearts undergo a gradual increase in susceptibility to TTX during early stages of development.

The results from the TTX experiments are consistent with the present findings that there may be two Na^+ inactivation potentials for hearts in the transition period. The slow channels are inactivated at a considerably lower resting potential compared to the fast channels. New fast Na^+ channels may be produced to replace the slow Na^+ channels, or each slow Na^+ channel may be converted into a fast Na^+ channel. The surface density of fast Na^+ channels may increase during the later part of development (e.g. days 8–18) because of the large increase in $+\dot{V}_{max}$ for about the same resting E_m. Much of the increase in P_K precedes the appearance of fast Na^+ channels. Preliminary experiments indicate that the fast Na^+ channels admit Li^+, whereas the slow Na^+ channels may not do so. It is not known whether the slow Na^+ channel

can admit Ca^{++}, or whether there may be a separate set of slow Ca^{++} channels. However, preliminary experiments indicate that ventricular cells of older embryonic hearts, rendered inexcitable by TTX, develop slow electrical responses upon addition of catecholamines which may be due to inward Ca^{++} current. It has been demonstrated that a TTX-insensitive slow channel system (blocked by Mn^{++}) is present in adult frog and rat myocardial cells; this system can carry Na^+ and/or Ca^{++}, and is activated and inactivated at lower E_m levels (Rougier et al., 1969; Garnier et al., 1969). Although we have not done voltage clamp experiments to get at the kinetics of the two populations of Na^+ channels, we have arbitrarily called one type slow, consistent with the definition of Coraboeuf and his collaborators, on the basis of: (*a*) its insensitivity to TTX, (*b*) its lower inactivation potential, and (*c*) the lower $+\dot{V}_{max}$ for the same takeoff potential. The present results, which suggest that there are only slow Na^+ channels in young hearts, cannot be explained on the basis of a low density of fast Na^+ channels (although the low $+\dot{V}_{max}$ could be explained by this), because: (*a*) of their lower inactivation potential and (*b*) of their insensitivity to TTX.

It is possible that the advent of cholinergic innervation may be related to the appearance of fast, TTX-sensitive Na^+ channels. Action potentials having large $+\dot{V}_{max}$ and sensitive to TTX first occur at about the period of cholinergic innervation. Gargouil and Bernard (1971) also recently reported that ventricles from rat embryos 10 days old (hearts first begin beating at 9–10 days) are insensitive to TTX, whereas those 13 days or older are sensitive; cholinergic innervation occurs between days 10 and 13. Rat skeletal muscle becomes insensitive to TTX a day or two after denervation, although the spike retains its dependency on Na^+ (but continues to be rather fast-rising) (Redfern and Thesleff, 1971). Cells taken from old (e.g. 16 days) chick embryonic ventricles lose their sensitivity to TTX and fire Na^+-dependent action potentials with much lower $+\dot{V}_{max}$ values when cultured in monolayers and thereby denervated (Sperelakis and Lehmkuhl, 1965; Sperelakis and Pappano, 1969); thus, the fast Na^+ channels of older embryonic myocardial cells are replaced with slow Na^+ channels when the cells are placed in culture. Myocardial cells in culture revert back to the early embryonic state in several other regards also: (*a*) P_K decreases, causing partial depolarization and enhanced automaticity. (*b*) the (Na^+, K^+)-ATPase specific activity is diminished (Sperelakis and Lee, 1971). (*c*) There is a loss of many myofibrils and some become disoriented (unpublished observations).

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