Developmental Changes in the Sensitivity of the Chick Embryo Ventricle to $\beta$-Adrenergic Agonist during Adrenergic Innervation

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SUMMARY

As early as the 4th embryonic day, the ventricle of the chick embryo responded to isoproterenol (Iso) with an increase in the force of contraction; at all ages studied, this positive inotropic effect was accompanied by an increased rate of tension development. There was a transient, 10-fold decrease in the sensitivity (increase in ED$_{50}$) of the right ventricle to Iso between the 16th and 21st embryonic day. This change in the sensitivity to Iso was not due either to an increased inactivation of Iso by non-neuronal cells or to a change in the thickness of the ventricle. It was found that adrenergic nerves were first capable of altering ventricular contractility on the 16th embryonic day. Whereas they interfered with the function of adrenergic nerves, injections of reserpine or 6-hydroxydopamine had no effect on the subsensitivity to Iso. Furthermore, these agents did not affect the normal developmental changes in heart weight. We conclude that the local release of transmitter from adrenergic nerves does not cause the transient subsensitivity of the ventricle of the chick embryo to $\beta$-adrenergic agonists.


DURING the formation of the neuromuscular junction of skeletal muscle, the postsynaptic skeletal muscle fiber undergoes a number of changes (reviewed by Vrbová et al., 1978). Before innervation, the muscle fiber begins to synthesize acetylcholine receptors and incorporates them into its plasma membrane in such a way that they are linked to functional ion channels. Around the time of innervation, there is a large change in the responsiveness to applied agonists which is accompanied by a change in the distribution of acetylcholine receptors and acetylcholinesterase activity. Innervation has also been found to exert trophic influences on other aspects of skeletal muscle development, such as the size of the muscle, the types of proteins synthesized, and the electrical properties of the muscle (Vrbová et al., 1978).

It is unclear to what extent analogous changes occur during the formation of adrenergic neuroeffector junctions. Usually postjunctional cells have been found to respond to adrenergic agonists before innervation (reviewed by Pappano, 1977; see also Furness et al., 1970). In many tissues, changes in sensitivity to adrenergic agonists have been seen during development (Frieswick et al., 1979; Furness et al., 1970; Pappano, 1977; Seidler and Slotkin, 1979; Standen, 1978; Stanton and Mersmann, 1979; Su et al., 1977). However, the possibility that there are postsynaptic sensitivity changes caused by adrenergic innervation has not been systematically explored. Adrenergic innervation has been found to affect the development in vivo of the ear artery (Bevan, 1975), and the intestinal epithelium (Klein and Torres, 1978). Under in vitro conditions, adrenergic nerves have been found to affect the state of differentiation of smooth muscle cells derived from blood vessels and from the vas deferens (reviewed by Burnstock, 1978). Indirect correlative evidence also has led some investigators to suggest that adrenergic innervation may also affect normal cardiac development by altering the synthesis of DNA (Claycomb, 1976), the development of sodium channels (Sperelakis and Shigenbou, 1974), and the synthesis of acetylcholinesterase (Taylor, 1977).

To develop a model in which to test the possibility that adrenergic innervation alters cardiac development, we have determined when adrenergic neuroeffector transmission first occurs in the embryonic avian heart and have begun to determine whether there are changes in some properties of cardiac muscle cells at this time. Particular attention has been given to the suspected role of adrenergic innervation in the regulation of the $\beta$-adrenergic sensitivity of the heart. In this regard, procedures that interfere with the ability of adrenergic axons to release their transmitter have been tested.

Methods

Fertilized eggs of White Leghorn chickens were obtained from SPAFAS and maintained under con-
ditions previously described (Pappano and Löffelholz, 1974).

Modified Tyrode’s solution (Pappano and Löffelholz, 1974) containing glucose (5.6 mm) and ascorbic acid (57 μM) was used for all superfusion experiments. Atropine sulfate (300 nM) was present in all experiments to antagonize the muscarinic effects of acetylcholine released from intraventricular parasympathetic cholinergic nerves (Pappano, 1977). All inhibitors were given 20 or more minutes before agonist challenges.

Cardiac Muscle Twitch Recordings

After the animal had been killed by decapitation, the heart was rapidly excised and placed in a tissue chamber (2.5 cm × 2.5 cm, 1 cm deep) that had a Sylgard (Dow-Corning) rubber floor. Under a dissecting microscope, the right ventricular free wall (RV) was isolated from embryos older than 9 days by cutting across the ventricular surface (just below the atrioventricular groove), and then around the contours of the RV. The bulbar regions and great vessels thus were excluded. (In the oldest animals, only a portion of RV was used so that the weight of tissue used never exceeded 15 mg.) For embryos younger than 8 days, both ventricles were isolated by a similar cut just below the atrioventricular groove. Experiments on 8- and 9-day embryos gave similar results with the RV or both ventricles, and so the results have been pooled. One end of the RV, usually the apical end, was pinned to the Sylgard floor and the tissue was superfused at 5–7 ml/min with modified Tyrode’s solution that previously had been gassed with 95% O₂, 5% CO₂. The temperature of the tissue chamber was maintained at 37 ± 1°C. To minimize mechanical interference, both the tissue chamber and the force-transducer were mounted on a Prior manipulator which, in turn, was stabilized by being attached to two large lead bricks. The free end of the tissue, usually the basal portion, was impaled with the capillary. Then the capillary tip and tissue were lifted slightly to avoid any scraping of the tip on the chamber floor. The tissue was stretched to the length that gave the greatest twitch tension. (This was not feasible in 4-day embryos, due to their mechanical fragility; at this age the ventricles were stretched only to length that gave a clear baseline signal.) The signal from the transducer was amplified (Grass 7P1B and 7DAE) and recorded on a Grass Polygraph or a Gould Brush 220 Recorder.

To avoid changes in force secondary to changes in rate, the heart was paced at 3 Hz. The punctate pacing cathode was a glass-insulated silver electrode with a tip diameter of about 250 μm: the diffuse anode was a platinum strip. The heart was paced by pulses that were 1.5 times threshold. (Typical threshold values were 0.5 msec duration pulses of 10 μA.) The tissue was allowed to equilibrate for 45 minutes before tests were begun.

Cumulative concentration-effect curves to isoproterenol (Iso) were obtained. The concentration which gave 50% of the maximum response (ED₅₀) was then graphically determined and used as an index of the sensitivity of the RV to Iso. Both arithmetic and geometric means and standard errors were computed. Since the method of computation did not affect the statistical significance (unpaired t-test unless otherwise noted), only arithmetic means are reported.

Intramural cardiac nerves were excited electrically by field stimulation (Pappano and Löffelholz, 1974). During equilibration, platinum electrodes were placed on either side of the tissue. Nerves were stimulated for 5 or 30 seconds with 50-mA monophasic pulses (5.0 msec in duration) of varying frequency. These parameters gave reproducible responses in older animals and caused only minor transient changes in twitch tension in an uninnervated RV and in a RV that had been treated with propranolol.

Injections

Three different methods of injection in ovo were used. Injections into the yolk sac were done by a modification of the method of Sparber and Shideman (1968). The end of the egg with the air space was wiped with 70% ethanol and a small hole over the air space was made by rotating a file the end of which had been sharpened. The egg was then placed on its side, a needle (23 gauge, 1”) was inserted parallel to the long axis of the egg, and a volume of 50 μl was injected. After the injection, the hole was covered with a small piece of Scotch tape.

Injections onto the chorio-allantoic membrane (CAM) were done by the method of Pittman et al. (1978). On the day of the first injection, a small area of shell over the air sac was removed and the part of the CAM was exposed by their procedure for partial membrane removal. Drugs were the placed (in a volume of 200 μl or less) on the exposed area. Subsequently, the hole in the egg shell was covered with Scotch Tape. The tape was removed for subsequent injections.

For intravenous injections, eggs were secured with clamp, and then, in a dark room, they were illuminated from the blunt end. Large vessels were located, the area over them was wiped with 70% ethanol, and then a small area of shell (about 1 cm × 1 mm) was removed. Injections were then made by hand with a 27-gauge (1”) needle. The volume
injected (50-200 μl) was rather large; this enabled us to verify the success of injections by noting whether the minor vessels under the shell momentarily blanched after the injection of the clear solution. Approximately half of the injections were successful. Some loss of blood was often noted about the site of the injection.

β-Receptor Histochemistry

9-Amino acridine-propranolol (9-AAP) and the dansyl analogue of propranolol (DAPN) were obtained from Polyscience Inc., on two separate occasions and were scrupulously shielded from light at all times after their receipt.

Animals were treated according to methods outlined either for visualization of β-receptors alone (Melamed et al., 1976) or for simultaneous visualization of β-receptors and catecholamines (Atlas and Segal, 1977). 9-AAP or DAPN (2 mg/10 ml) was administered in a 50% ethanol solution by slow intravenous injection. Thirty minutes after drug injection, the animals were killed by decapitation.

Results

Developmental Changes in the Sensitivity of Ventricular Muscle to β-Adrenergic Agonists

Iso was used to stimulate β-adrenergic receptors. From the 4th embryonic day onward, paced ventricular muscle responded to Iso (1 μM) with a prompt, reversible increase in the force of contraction. This positive inotropic response was apparent within 15 seconds of the entry of the drug into the tissue chamber and was maximal in less than 3 minutes. A typical record illustrating the response of the ventricles of a 4-day embryo to Iso is reproduced in Figure 1; similar results were obtained in four of four experiments with ventricles from 4-day embryos and in two of two experiments with 5-day embryos. The magnitude of the increase in force (as a percent of basal tension) in 4-day embryos was smaller than that seen in older embryos (compare Figs. 1 and 2). Because of a poor signal-to-noise ratio in preliminary experiments, we did not try to determine whether there was a positive inotropic response to Iso in ventricles from embryos younger than 4 days.

In adult hearts, β-adrenergic agonists characteristically exert their effects on twitch tension by increasing the rate of tension development while decreasing both the time-to-peak tension (TPT) and the duration of the contraction (Tsien, 1977). Signals from ventricles of 5-day and older embryos were differentiated electronically. The increase in the force of contraction in the presence of Iso was accompanied at all ages by an increase in rate of tension development; the concentration-effect curves of these two changes were virtually superimposable (Fig. 2). In most experiments, Iso had no effect on either the TPT or the duration of the contraction at any age tested. However, in some experiments, Iso shortened both. The response to Iso was antagonized by propranolol (300 nM) but unaffected by phentolamine (10 nM) or atropine (300 nM).

Cumulative concentration-effect curves to Iso were obtained from the RV from the 8th embryonic day to the 7th day after hatching; the ED₅₀ was used as an index of the sensitivity to Iso. The sensitivity to Iso was constant from the 8th to the 14th embryonic day (Fig. 3). During the next few days, the RV became less sensitive to ISO; the ED₅₀

![Figure 1](Positive inotropic effect of Iso (1 μM) in embryonic ventricles from a 4-day chick embryo. At the arrow, superfusion with Iso began. Time marks at 1-second intervals show the constant rate of contraction (3 Hz). The recording speed was reduced 60-fold before and after Iso infusion. The average twitch tension was 4.8 ± 0.1 mg (n = 3) before addition of Iso (see Discussion for limitations).)

![Figure 2](Concentration-effect curve for Iso in the RV from a 12-day embryo. Abscissa: concentration of Iso (nM). Ordinate: peak twitch tension (●) and maximal rate of development of twitch tension (▲) as a percent of control. The mean twitch tension was 188 ± 33 (n = 4) mg before addition of Iso (see Discussion for limitations).)
rose from 5.3 nM on day 14 to 38 nM on day 17. This decrease in sensitivity was rapid; a 3-fold change occurred between the 16th and 17th day in ovo. The subsensitivity to Iso was also transient. On the 21st day (day of hatching), the ED50 for Iso had fallen to 7.9 nM: 1 week after hatching, the RV was as sensitive (ED50 = 2.3 nM) as it had been during the 2nd week in ovo.

Although Iso is not transported by adrenergic nerves, it can be taken up by non-neuronal cells and subsequently metabolized (Trendelenburg, 1978). Corticosterone (30 μM) has been found to inhibit this uptake process by more than 90% in the rodent heart (reviewed by Trendelenburg, 1978). In 18-day embryos, the ED50 for Iso was not significantly different in the presence (36 ± 9 nM, n = 3) or absence (35 ± 5 nM, n = 12) of 30 μM corticosterone.

β-Receptor Histochemistry

Melamed et al. (1976) have reported that cardiac β-receptors of adult rats can be labeled with fluorescent analogues of propranolol (9-AAP and DAPN) and then visualized in the fluorescence microscope. We attempted to replicate these experiments and to extend them to hatched chicks. Eight animals (five adult Sprague-Dawley rats and three chickens) received injections of either 9-AAP (2.5 to 8.5 mg/kg) or DAPN (2.5 to 7.5 mg/kg); control animals (no injection, saline injection or 9-aminoacridine) were processed in parallel with experiments. The submandibular gland (rats only), atria, and ventricles were examined. In no experiment could we detect a difference between the appearance of tissue sections from control animals and of sections from experimental animals. In salivary glands not prepared for catecholamine histochemistry, the only fluorescent structures seen (aside from a faint green background) were small (about 1 μm), very faint, yellowish to yellowish-orange granules. They appeared to be more common over acinar cells. However, they were also present in control animals. In cardiac tissue not processed for catecholamine histochemistry, some faint yellowish granules (~1 μm) were found scattered through the sections. They were equally abundant in experimental and control groups.

Relationship of Postjunctional Sensitivity Changes to Innervation

Adrenergic nerves first are capable of altering cardiac contractility on the 16th embryonic day (Higgins, 1980). Since this was the day on which the sensitivity to Iso began to decrease, it seemed possible that the subsensitivity was caused by the release of transmitter from newly formed cardiac adrenergic nerves. To test this hypothesis, we attempted to delay the onset of neuroeffector transmission by the administration in ovo of two agents, reserpine and 6-hydroxydopamine (6-OHDA), which impair adrenergic function.

Because of its long-lasting effects in ovo (Discussion) embryos received only one injection of reserpine (10 μg in 50 μl/egg; 1 egg = 60 g). Reserpine was injected into the yolk; the vehicle was 10% ascorbic acid. Embryos tested on the 14th day had been injected on the 7th day; embryos tested on or after the 17th day had been injected on day 11. The effects of injections of reserpine and its vehicle on embryo survival and hatchability are shown in Table 1. In uninjected embryos, mortality in ovo was less than 4%, and more than 90% eventually hatched. Vehicle injections on day 11 did not affect survival; similar injections on day 7 increased mortality only slightly. Injections of reserpine on day 11 markedly depressed hatchability (28% vs. 94%) while only slightly increasing mortality in ovo. Injection of the same dose of reserpine into 7-day embryos caused mortality to rise to 54%.

Reserpine injections in ovo effectively delayed the onset of adrenergic neuroeffector transmission (Fig. 4). For example, when intracardiac adrenergic nerves were electrically excited by field stimulation for 5 seconds on the 18th embryonic day, the RV responded with an increase in force equivalent to 44% of the increase obtained in response to 1 μM Iso. In reserpine-treated embryos, the response to nerve stimulation was only 3% of the response to Iso. From the data in Figure 4, it is clear that reserpine continued to depress profoundly nerve
function through the day of hatching (day 21), whereas vehicle injections had no statistically significant effect. Since reserpine did not alter the growth of the embryo (Table 2) or of the embryonic heart, it is unlikely that it was acting non-specifically to retard development.

The developmental changes in the ED₅₀ for Iso in embryos treated with reserpine are shown in Table 3. Injections of reserpine altered neither the magnitude nor the time course of the subsensitivity to β-agonist. Similarly, the maximum increase in twitch tension obtained in the presence of Iso on the 18th embryonic day was the same in control animals (225 ± 23% of basal tension, n = 10) and in animals that had received injections of reserpine (244 ± 33%, n = 4).

Preliminary experiments revealed that a single intravenous injection of a high dose (100 mg/kg egg) of 6-OHDA on the 13th embryonic day had only a small effect on adrenergic function measured 5 days later (Table 4). Therefore, it seemed necessary to give at least two injections. Because of the high mortality (Table 1) associated with intravenous injections in ovo, we tried other methods of introducing 6-OHDA into chick embryos. After a series of two injections (days 14 and 16) of 6-OHDA onto the CAM (Table 4), adrenergic nerve function on the 18th day appeared to be depressed under these conditions; however, the effects of 6-OHDA did not seem to be as great or as consistent as the effects of reserpine. Preliminary experiments with two injections into the yolk sac caused an impairment of adrenergic function comparable to that seen with CAM injections (Table 4).

The mortality was high with all methods of 6-OHDA injection (Table 1). However, high doses were needed to interfere with adrenergic function. In embryos receiving a total of 200 mg/kg, small, dark deposits of oxidation products could be seen near the shell membrane; the yolk and liver also were discolored. Surprisingly, in the embryos that survived this treatment, heart weights were normal (Table 2).

The development of subsensitivity to Iso was not prevented by injections of 6-OHDA (Table 4); in fact, the RV was even less sensitive than control preparations.

Discussion

It has been proposed that β-adrenergic agonists increase the force of contractions of the adult heart, at least in part, by causing an increase in intracellular cAMP which, in turn, leads to an increase in the size of a slow inward current carried largely by Ca²⁺ (reviewed by Tsien, 1977). Specific binding of the β-adrenergic ligand dihydroalprenolol has been

### Table 1  Effects of Injections of Reserpine or 6-OHDA on Embryo Survival

<table>
<thead>
<tr>
<th>Condition</th>
<th>Embryonic day</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>7</td>
</tr>
<tr>
<td>No injection</td>
<td>68/68</td>
</tr>
<tr>
<td>10% ascorbic acid on day 7</td>
<td>21/21</td>
</tr>
<tr>
<td>10% ascorbic acid on day 11</td>
<td>18/18</td>
</tr>
<tr>
<td>Reserpine on day 7</td>
<td>28/28</td>
</tr>
<tr>
<td>Reserpine on day 11</td>
<td>40/40</td>
</tr>
<tr>
<td>6OHDA iv</td>
<td>14/14</td>
</tr>
<tr>
<td>6OHDA (into yolk)</td>
<td>6/6</td>
</tr>
<tr>
<td>6OHDA (onto CAM)</td>
<td>27/27</td>
</tr>
</tbody>
</table>

The numerator is the number of embryos surviving; the denominator is the total number of embryos observed. Doses are given in the text.
detected in the 4.5-day embryonic heart (Alexander et al., 1978). β-Agonists usually have been found to increase cAMP in the 4-day embryo heart (reviewed by Pappano, 1980). Biegon and Pappano (1980) have reported that, as early as the 3rd embryonic day, Iso increases the inward current carried by a kinetically slow ion channel. It would thus appear that, by the 4th embryonic day, many of the proposed intermediate mechanisms linking β-agonists to increases in twitch tension are operative.

Frieswick et al. (1979) observed a positive inotropic response to Iso in hearts of chick embryos on the 3rd and 4th embryonic days; such a response was not detected by other investigators (reviewed by Pappano, 1977, 1980). Our results suggest that, in a preparation where the rate was clearly controlled, β-agonists can increase contractility as early as the 4th embryonic day and that, as reported by Frieswick et al. (1979), this change is due to a direct effect on ventricular muscle. The cause of these discrepant results is not clear. However, the increases in tension reported in this study (about 33%) and in the study by Frieswick et al. (1979) (about 50%) are small; in older animals a several-fold increase in tension is common. On the 4th embryonic day, the heart is small [less than 800 mg (Romanoff, 1960)], very fragile, and its contractions are very weak. Therefore, it is difficult to obtain records in which the signal-to-noise ratio is great enough to detect small changes in twitch tension. Furthermore, it should be noted that the detection of β-agonist-induced increases in contractility in hearts of adult animals can be hampered by the choice of an inappropriate external calcium concentration, frequency of stimulation (Bereswicz and Reuter, 1977), or experimental temperature (Blinks and Koch-Weser, 1963).

In adult animals, β-adrenergic agonists have several characteristic actions on cardiac muscle twitches (Tsien, 1977). The most consistently observed effect is to increase the rate of tension development; this also occurs in the developing ventricle at least as early as the 5th embryonic day. In the adult heart, β-agonists usually shorten both TPT and twitch duration; no consistent change in either parameter was observed on the 8th or 14th embryonic day. Our inability to detect such changes probably is related to the fiber geometry of the preparation. The geometric relationships among muscle fibers in the ventricular wall are complex (Blinks and Koch-Weser, 1963); unlike fibers in a papillary muscle, they are not all aligned parallel to an axis. Although we attempted to use areas of the RV where the superficial fibers were roughly parallel, there may have been sufficient distortion of our mechanical recordings to obscure a small change in TPT. When papillary muscles from mature chickens were examined, it was possible to

<table>
<thead>
<tr>
<th>Age in ovo (days)</th>
<th>Treatment</th>
<th>Heart wt (mg)</th>
<th>Embryo wt (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>14</td>
<td>None</td>
<td>110 ± 7 (8)*</td>
<td>9.8 ± 0.5 (5)</td>
</tr>
<tr>
<td></td>
<td>Ascorbic acid</td>
<td>103 ± 5 (6)</td>
<td>8.9 ± 0.7 (4)</td>
</tr>
<tr>
<td></td>
<td>Reserpine</td>
<td>108 ± 4 (6)</td>
<td>10.1 ± 0.7 (4)</td>
</tr>
<tr>
<td>18</td>
<td>None</td>
<td>228 ± 9 (9)</td>
<td>24.1 ± 0.6 (5)</td>
</tr>
<tr>
<td></td>
<td>Ascorbic acid</td>
<td>232 ± 18 (4)</td>
<td>23.4 ± 1.0 (4)</td>
</tr>
<tr>
<td></td>
<td>Reserpine</td>
<td>210 ± 10 (3)</td>
<td>24.0 ± 0.9 (4)</td>
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<tr>
<td></td>
<td>6-OHDA (onto CAM)</td>
<td>234 ± 9 (5)</td>
<td></td>
</tr>
<tr>
<td>19</td>
<td>None</td>
<td>250 ± 41 (4)</td>
<td>24.3 ± 0.6 (7)</td>
</tr>
<tr>
<td></td>
<td>Ascorbic acid</td>
<td>240 ± 23 (4)</td>
<td>25.5 ± 0.5 (3)</td>
</tr>
<tr>
<td></td>
<td>Reserpine</td>
<td>238 ± 39 (4)</td>
<td>25.2 ± 0.6 (3)</td>
</tr>
</tbody>
</table>

Results are expressed as mean ± SE. Neither drug treatment had a statistically significant effect. Doses are given in text.
demonstrate a decrease in TPT in the presence of Iso (Higgins and Pappano, unpublished observations).

Since adrenergic nerve terminals could not be found in the chick embryo RV before the 11th day (Higgins and Pappano, 1979), it is clear that ventricular muscle cells can respond to transmitter before innervation. Not all components of the β-adrenergic response have been examined (Tsien, 1977); however, within the limits of the assay systems used, it appears that the biochemical, electrical, and mototropic responses to β-agonist are qualitatively similar in early embryonic and adult hearts. In contrast, the response of the embryonic heart to its other transmitter, acetylcholine, has been found to change qualitatively during development (Pappano, 1977). On the 3rd embryonic day, acetylcholine causes a depolarization of chick sinoatrial cells and only temporarily arrests the heart. On the 18th embryonic day, it causes a hyperpolarization and its negative chronotropic effect is persistent.

Whereas there were no qualitative changes in the response to β-agonist, there was a transient quantitative (10-fold) change in the sensitivity to Iso during the 3rd week in ovo. Although adrenergic nerves appear in the heart at this time, it is unlikely that they directly altered the metabolism of Iso. Isoproterenol has been found not to be taken up by adrenergic nerves in a number of tissues of various species (reviewed by Trendelenburg, 1979). Ignarro and Shideman (1968) found the activity of catechol-O-methyltransferase rose transiently in the chick embryo heart during the 3rd week in ovo. Since inhibition of this enzyme sensitizes the cat heart to Iso (Trendelenburg, 1979), it seemed possible that the change in sensitivity to Iso was due to increased uptake and metabolism of Iso by non-neuronal cells. However, corticosterone, an inhibitor of non-neuronal Iso uptake in a variety of mammals (Trendelenburg, 1978), did not affect the ED₅₀ for Iso. Assuming that corticosterone acts similarly in birds, it can be concluded that the subsensitivity to Iso is not due to inactivation by either neuronal or non-neuronal cells. Whereas changes in the accessibility of β-receptors cannot be excluded, it is unlikely the subsensitivity is due to changes in the tissue size, since the RV grows thicker throughout the stages studied. However, the sensitivity to Iso increased after the 19th embryonic day; it was as great on the 7th day after hatching as it was on the 8th embryonic day. It appears probable that the subsensitivity to Iso is due to a change in the postjunctional, that is, ventricular muscle cells.

Spare receptor theory (Williams and Lefkowitz, 1978) predicts that a change in sensitivity to β-agonists can be caused by a change in the density of β-adrenergic receptors. In agreement with this theory, changes in sensitivity to β-agonists in many tissues (Williams and Lefkowitz, 1978) have been accompanied by changes in the number of specific β-adrenergic ligand binding sites. In the chick embryo heart, Alexander et al. (1978) have measured the binding of dihydroalprenolol (DHA), a β-adrenergic ligand. They found a 32% decrease in DHA binding per milligram protein between the 9th and 13th embryonic day. There was no change in the number of binding sites per milligram of protein between the 13th and 17th day, the period during which a large change in the sensitivity to Iso was observed. It has been observed that, in the swine heart, there is a similar lack of correlation between developmental changes in sensitivity to Iso and changes in the density of DHA-binding sites (Stanton and Mersmann, 1979). These results are not consistent with the possibility that the change in sensitivity to Iso was caused by a change in the density of β-adrenergic receptors. However, there are developmental changes in the amount of protein per gram tissue (Ignarro and Shideman, 1968; Romanoff, 1960), in the amount of myosin per myocyte (Manasek, 1979), and in the cellular composition of the heart (Manasek, 1979) which make it difficult to interpret the significance of developmental changes in the amount of DHA bound by a milligram of protein.

When the neuromuscular junction of voluntary muscle is formed, there is a change in the distribution of acetylcholine receptors and in the sensitivity to applied cholinergic agonists (Vrbová et al., 1978). Melamed et al. (1976) reported that β-receptors were not distributed uniformly on cardiac muscle cells; rather, clusters were observed. It seemed possible some of the change in sensitivity to Iso might

### Table 4

<table>
<thead>
<tr>
<th>Injection method</th>
<th>No. of injections</th>
<th>Amount of injection</th>
<th>Days of injection</th>
<th>ΔA/ΔB</th>
<th>Iso ED₅₀ (nm)</th>
</tr>
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<tr>
<td>None</td>
<td>0</td>
<td>0</td>
<td>13</td>
<td>0.44 ± 0.04</td>
<td>35 ± 5(12)*</td>
</tr>
<tr>
<td>iv</td>
<td>1</td>
<td>100 mg/kg</td>
<td>11, 15</td>
<td>0.30 ± 0.03</td>
<td>30 (1)</td>
</tr>
<tr>
<td>Into yolk</td>
<td>2</td>
<td>100 mg/kg</td>
<td>14, 16</td>
<td>0.08 ± 0.05</td>
<td>76 ± 5(5)</td>
</tr>
<tr>
<td>CAM</td>
<td>2</td>
<td>100 mg/kg</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Results are expressed as mean ± SE. Embryos were injected by various methods with 6-OHDA on the day(s) shown in the table. Adrenergic nerve function was tested on the 18th embryonic day. The ratio ΔA/ΔB has been previously defined (Fig. 4 and text). The vehicle was 0.9% NaCl with 0.2 mg/ml ascorbic acid. The volume was 100 μl/injection.

* Number.
be due to a change in the distribution of $\beta$-receptors at the time of adrenergic innervation. We attempted to visualize $\beta$-receptors, but were unable to detect any specific fluorescence. Hess (1979) has reported a similar lack of success in neural tissue and has suggested that the fluorescence previously attributed to $\beta$-receptors may have been due to autofluorescent granules. Such granules have also been reported in dog heart (Dahalstrom et al., 1965).

In adult animals, reduction of adrenergic neural input has been found to affect postjunctural sensitivity to catecholamines in some organs, such as the nictitating membrane (Trendelenburg, 1963). This model may also apply to cardiac muscle (Meisher et al., 1979). It has been shown that adrenergic nerves are first capable of altering cardiac contractility on the 16th embryonic day (Fig. 4 and Higgins, 1980). Since this was the day on which the sensitivity to Iso began to decrease, it seemed possible that the release of catecholamines from newly formed cardiac adrenergic nerves caused the subsensitivity to Iso. Therefore, we tried to delay the onset of neuroeffector transmission.

Single injections of reserpine in ovo have been found by others to chronically lower tissue catecholamine levels (Pittman et al., 1978). Higgins (1980) reported that reserpine in ovo also chronically depresses the ability of cardiac adrenergic nerves to accumulate or release $^3$H-norepinephrine. In assays of function, reserpine abolished the ability of adrenergic nerves to alter cardiac contractility at any time in ovo. Reserpine had no effect on the time course of the changes in sensitivity to Iso; therefore, the local release of catecholamines by adrenergic nerves did not cause the subsensitivity to Iso. Perhaps the mechanism of the regulation of $\beta$-adrenergic sensitivity in the RV of chick embryo is different from that in the brain of the neonatal rat (Harden et al., 1979).

Consistent with this hypothesis, 6-OHDA interfered with adrenergic nerve function, yet did not block the transient subsensitivity to Iso. However, the 6-OHDA-treated RV was even less sensitive to Iso than the control RV on the 16th day. When large amounts of it are used, 6-OHDA has been found to react with catecholamine binding proteins such as postjunctional $\alpha$-adrenergic receptors and the neuronal catecholamine transport system (Sachs and Jonsson, 1975). Since large doses were injected into a closed system, it is possible that 6-OHDA affected another catecholamine-binding protein, the $\beta$-adrenergic receptor, in the embryonic heart. Two rather large doses of 6-OHDA were needed to depress neuroeffector transmission. Although a systematic investigation was not undertaken to explain this result, there are two observations in developing animals that are relevant. First, Kirby (1978) observed that the appearance of sympathetic ganglia in the chick embryo was not altered by injections of 6-OHDA. Second, whereas 6-OHDA is usually sympatholytic in developing animals (Sachs and Jonsson, 1975), it has also been noted to stimulate nerve growth and cause an anomalous hyperinnervation in the central nervous system (Harden et al., 1979).

The transient subsensitivity to catecholamines is widespread in the chick embryo; it has been seen in the RV, sinus node, and vascular bed (reviewed by Pappano, 1977). It seems possible that changes in levels of circulating catecholamines could modulate adrenergic sensitivity. However, no data on plasma levels of catecholamines in chick embryos are available and data from other developing systems are conflicting (Behrens et al., 1979; Pappano, 1977). Alternatively, there are transient changes in the levels of many endocrine hormones during development (Freeman and Vincen, 1973). Since chronic administration of either thyroid hormone or corticosteroids (Williams and Lefkowitz, 1978) has been found to alter sensitivity to catecholamines in mature animals, the changes in Iso sensitivity may be mediated hormonally. Again, it is possible that peripheral nerves may release substances other than neurotransmitters which alter the development of the postjunctional cell (Vrbová et al., 1978).

Claycomb (1976) proposed that adrenergic innervation influenced protein and DNA synthesis in the heart. Adrenergic neuroeffector transmission was first observed in the chick embryo RV on the 16th embryonic day. By this time, the rate of DNA synthesis in the heart has already fallen to low levels (Doyle et al., 1974). Furthermore, during a period when heart weight nearly doubled, neither of two agents (reserpine and 6-OHDA) which interfered with adrenergic transmission prevented a normal increase in heart weight. Similar results have been reported by Lau et al. (1979).

Thus, it can be concluded that the chick embryo RV can respond to $\beta$-agonists before innervation, that the response is qualitatively the same throughout development, and that there is a transient change in sensitivity to $\beta$-agonist which is not caused by local release of transmitter from adrenergic nerves at the onset of neuroeffector transmission.

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