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# Development of heart rate irregularities in chick embryos

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**Höchel, Joachim, Ryuichi Akiyama, Takuya Masuko, James T. Pearson, Martin Nichelmann, and Hiroshi Tazawa.** Development of heart rate irregularities in chick embryos. *Am. J. Physiol.* 275 (*Heart Circ. Physiol.* 44): H527–H533, 1998.—Heart rate (HR) irregularities in chick embryos were defined as large fluctuations (>10 beats/min) comprising irregular, brief deceleration and/or acceleration of instantaneous HR (IHR). IHR was determined directly from the arterial blood pressure while adequate gas exchange was maintained through an eggshell and chorioallantoic membrane. Five embryos were examined on each day from *day 11* to *day 19* of incubation. Baseline HR was stable until *day 12–13*, and on around *day 13–14* transient, rapid deceleration of HR (termed *V* pattern) began to appear, with a subsequent increase in its frequency and magnitude. The acceleration patterns (lambda, avian omega, and periodic patterns) appeared later, and the IHR became increasingly irregular, with additional, spontaneous deceleration and acceleration patterns toward hatching. Additional experiments with intravenous administration of autonomic drugs clearly showed that rapid deceleration of HR was mediated by parasympathetic nervous function but did not always show clear relations of sympathomimetic and sympathetic blocking agents to the acceleration patterns.

blood pressure of allantoic artery; instantaneous heart rate; deceleration and acceleration patterns; heart rate fluctuations and variability; autonomic drugs

OVER THE LAST 10 years there has been considerable interest in heart rate (HR) fluctuations in relation to the autonomic nervous system. Although the beat-to-beat HR fluctuations are known as HR variability (HRV), which tends to be oscillating and rhythmic and has been subject to considerable attention, large fluctuations comprising irregular, brief decelerated and/or accelerated HR may be termed HR irregularities (HRI). HRI have been reported in the fetus and consist of several patterns (2, 3, 6, 12, 15, 28), and they seem to be a substantial phenomenon in developing animals. The chick embryo has been used as an experimental animal model to study developmental physiology (4). While working on techniques to measure physiological functions of avian embryos within an eggshell, we measured noninvasively instantaneous HR (IHR) of developing chick embryos by taking advantage of an acoustocardiogram (ACG) and found HRI, the occurrence of which in avian embryos had been suggested by measurements of ballistocardiogram (BCG) and electrocardiogram (1, 13, 20, 22). Although the ACG method has an advantage of noninvasive, long-term measurement of embryonic HR and showed the development of

HRI during continuous HR recording throughout the last half of incubation (1), a complementary method is needed to substantiate in detail the HRI patterns, because unknown artifacts occasionally interfered with ACG waves. In chicken eggs the allantoic artery can be catheterized through a small hole in the eggshell, and the arterial blood pressure can be measured by a conventional electromanometer (17, 18). Although the blood pressure measurement is made invasively and acutely, we used it to determine the IHR and to investigate the HRI patterns in developing chick embryos. In additional experiments the allantoic vein was also catheterized for administration of autonomic drugs, and their effects on the HRI were examined.

## MATERIALS AND METHODS

Fertile eggs of broiler chickens were brought from a local hatchery to the laboratory, incubated at 37.5°C and 60% relative humidity in an incubator, and turned four times daily. Timing of the eggs started when they were put in the incubator, and the 1st day was designated *day 0* of incubation. The total incubation period of the chicken eggs was 21 days. Catheterization of the allantoic artery was attempted in the prenatal embryos (referred to as embryos) after the first half of incubation until the perinatal embryo (referred to as chick fetus) pierces the air cell with its beak. Thus the present blood pressure measurement was not made in chick fetuses. A 26-gauge 15-mm-long hypodermic needle attached to a 5-cm-long polyethylene tube was used as a catheter (18). The needle was bent at a right angle 2–3 mm from the tip. The catheter was filled with heparin solution to prevent blood clotting, and the free end of the tube was plugged with clay. Because the gas exchange of embryos takes place by molecular diffusion through the pores of the eggshell, the implantation of the catheter into the allantoic artery was made with minimal impediment to gas exchange through the eggshell and chorioallantoic membrane (CAM), as described in previous reports (17, 18). Briefly, a small area, marked previously on the shell through candling (<1 cm<sup>2</sup>), was removed with the shell membranes, and the allantoic artery was lifted by forceps from the allantoic fluid through a tear in the CAM. The tip of the catheter was inserted into the artery pointing upstream. After the catheterized artery was repositioned in the allantoic fluid, the catheter was fixed to the edge of the hole in the shell, and the hole was recovered with tape and epoxy. The catheterized egg was replaced in the incubator and warmed for 2–3 h for thermal equilibration. For measurement, the egg was transferred to a small chamber in the same incubator, and the chamber containing the egg was vented with air. The polyethylene tube emerging from the egg was connected to an electromanometric transducer (model P23ID, Statham) through another polyethylene tube filled with saline solution. The transducer was placed at the same height as the egg, and the pressure signals were amplified by a

polygraph amplifier to match the input signal level of an analog-to-digital converter. The pressure signals were sampled at 100 Hz, recorded on a microcomputer, and restored by a sinc function, as described previously for the ACG signal (1). The wave restoration by the sinc function by use of 401 sampling points corresponded to a signal sampled at 8,000 Hz, ensuring calculation of IHR with an error in accuracy of  $<1$  beat/min. After restoration of the systolic pressure wave, the maximum point was found in the restored wave, the time interval between the two adjacent maximum points was determined, and IHR was calculated from the time interval.

In additional eggs we also attempted to implant the catheter into the allantoic vein, with a procedure similar to that used for implantation into the allantoic artery. The volume of the venous catheter was  $\sim 50$   $\mu$ l (i.e., dead space for infusion of drugs). For administration of autonomic drugs, the plugged end of the catheter was cut off, the needle of a syringe containing a drug was inserted, and a plunger was pulled back slightly to ensure that the catheter remained in the vein, in which case blood could be seen in the catheter. Ten to 50  $\mu$ l of solution, depending on dose, were administered. Soon after this, 50  $\mu$ l of saline solution were infused from another syringe, and the end of the tubing was plugged again with clay. The following drugs were examined for their effects on the HRI: ACh (parasympathomimetic drug), atropine (parasympathetic blocking agent), norepinephrine and isoprenaline (sympathomimetic drugs), phentolamine ( $\alpha$ -adrenergic blocking agent), and propranolol ( $\beta$ -adrenergic blocking agent). In these drug experiments, arterial blood pressure was measured in the embryos during the first 30 min to obtain control HRI and during the next 1 h after administration of a drug.

## RESULTS

Implantation of the catheter was attempted from *day 10* of incubation. However, only one egg was catheterized, and arterial blood pressure was measured for 2.5 h on *day 10*. From *day 11* it became possible to implant a catheter in most eggs in which implantation was attempted. However, the catheterization was not always successful in all eggs, and even if it was successful, in some embryos the arterial blood pressure dropped suddenly because of the catheter's escape from the artery or bleeding. Such blood pressure recordings were discarded. Five embryos whose blood pressure was measured successfully without distortion for  $>30$  min were used for investigation of HRI on each day from *day 11* to *day 19* of incubation. After *day 19* it became difficult to implant the catheter because of natural atrophy of the allantoic vessels after internal pipping, and the catheterization was discontinued. One embryo that was catheterized on *day 19* was subjected to measurement from *day 19* to *day 20*. The arterial blood pressure of the 46 eggs, including 1 egg on *day 10*, was monitored on an oscilloscope for as little as 1 h to  $>2$  days, until the pressure signals deteriorated. Blood pressure was recorded on the computer intermittently during these periods.

Figure 1 shows typical 30-min recordings of IHR in 11- to 20-day-old embryos. The mean HR and mean blood pressure of all five embryos on each day of incubation are presented in Table 1. For mammalian fetal HR patterns, HR changes of  $\leq 10$  beats/min were referred to as baseline HRV. Larger deviations from the

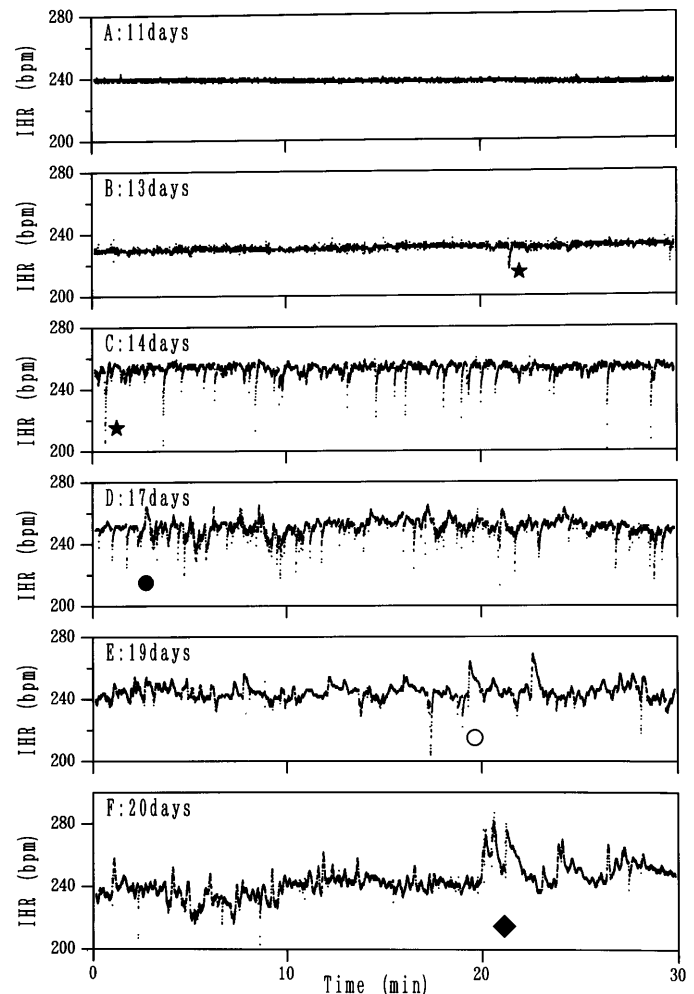


Fig. 1. Instantaneous heart rate (IHR) recorded from 5 embryos on *days 11, 13, 14, 17, 19, and 20* of incubation:  $237 \pm 1$  ( $n = 7,079$ ),  $230 \pm 1$  ( $n = 6,874$ ),  $252 \pm 4$  ( $n = 7,522$ ),  $250 \pm 5$  ( $n = 7,455$ ),  $244 \pm 5$  ( $n = 7,270$ ), and  $243 \pm 9$  (SD) beats/min ( $n = 7,245$ ), respectively. IHR values are plotted as individual points. IHR on *days 19 and 20* were recorded from same embryo.  $\star$ , Rapid deceleration;  $\bullet$ , transient increase followed by transient decrease with slower recovery to baseline;  $\circ$ , rapid increase followed by slower recovery to baseline without transient decrease;  $\blacklozenge$ , periodic pattern.

baseline were HRI, if they were of short duration ( $<1$  min). On *day 11* of incubation (Fig. 1A), HRV was small in the embryo illustrated, as in all the other embryos. On the following day, IHR tended to fluctuate intermittently, and the baseline variability became distinguished, but no embryos showed any particular pattern of HRI. On *day 13* (Fig. 1B), small rapid decreases in HR occurred up to several times during the 30-min recordings. Frequency and magnitude of such rapid deceleration patterns depended on recording time and also on individual embryos. On the following day (Fig. 1C) the spontaneous rapid deceleration patterns appeared with increasing frequency and magnitude. After *day 14* the HRI were augmented by additional irregular patterns. Four patterns of HR changes were distinguished.

Figure 2 presents the four patterns of HRI and their respective systolic and diastolic blood pressures. Figure

Table 1. Mean heart rate and blood pressure with embryos on each day of incubation

	Day of Incubation								
	11	12	13	14	15	16	17	18	19
Mean HR, beats/min	221 ± 10	244 ± 8	244 ± 17	251 ± 10	250 ± 15	248 ± 8	250 ± 2	240 ± 6	239 ± 6
Mean BP, mmHg									
Systolic	8.7 ± 1.1	12.4 ± 1.1	13.4 ± 2.4	16.7 ± 3.1	18.2 ± 2.6	20.8 ± 2.3	25.3 ± 4.0	26.7 ± 6.2	31.6 ± 3.3
Diastolic	5.6 ± 1.3	7.6 ± 0.7	8.4 ± 2.5	10.4 ± 2.3	10.5 ± 2.1	11.9 ± 2.4	16.1 ± 2.8	15.3 ± 5.1	19.8 ± 2.0

Values are means ± SD averaged for first 30 min of recording for 5 embryos. HR, heart rate; BP, blood pressure.

2A shows a pattern that was characterized by a sudden, rapid decrease followed by a slower recovery to baseline within ≤10 s. HRV also occurred, making it difficult to determine the baseline. The nadir was reached within a few heartbeats, and there were many cases in which it was reached within one or two heartbeats. This pattern was termed a *V* pattern, which is similar to findings in human fetuses during the second trimester of gestation (12, 15, 28) and prevalent in chick embryos after about day 14. Diastolic pressure decreased slightly (~1 mmHg) in association with rapid deceleration in HR. The pattern in Fig. 2B was characterized by a transient increase followed by a transient decrease with a slower recovery to baseline within 30 s. This pattern was also reported in human fetuses and termed a lambda pattern (2, 3). It began to appear on day 15–16 in chick embryos, making the HR more irregular with embryonic development. The systolic and diastolic pressures tended to increase, but the increase was not large. Figure 2C shows a rapid increase followed by a slower recovery to baseline without a transient decrease. Although this pattern was different from an omega pattern (i.e., a transient increase) reported in the human fetus (2) with respect to a faster increase, both patterns were similar in terms of a transient increase with slower recovery. Therefore, we termed this pattern an avian omega pattern. The lack of the transient decrease in the omega pattern differed from the lambda pattern. The systolic and diastolic pressures increased slightly in association with the rapid increase in HR and then returned to the original level while the HR still increased. The periodic pattern in Fig. 2D was a succession of avian omega patterns as reported in the fetus as a periodic pattern (2).

In addition to these four basic patterns, which were similar to those reported in the human fetus, outstanding single-beat changes in HR could be seen. Figure 3 shows two additional patterns, a single-beat acceleration followed by a single-beat deceleration and an acceleration followed by a transient decrease with a single-beat deceleration, together with the blood pressure recording corresponding to them. They may be termed a single-beat lambda pattern and a lambda pattern with a single-beat deceleration, respectively. Besides these distinguishable patterns, spontaneous decelerations and accelerations of short duration appeared frequently, particularly during the late stages of incubation.

Additional catheterization of the allantoic vein was attempted in 14- to 17-day-old embryos. It was difficult to maintain two catheters in the artery and vein in

embryos during drug administration experiments, and many embryos died during these experiments. Thus it was difficult to collect the IHR data showing the effects of the autonomic nervous system on the HRI. However, it was manifestly proven that the rapid, transient deceleration (*V* pattern) of IHR was mediated by the parasympathetic nerve. Figure 4 presents IHR responses to administration of autonomic drugs. Before drug administration experiments, we certified that infusions of 50 µl of saline solution did not produce marked changes in HRI recordings. Figure 4A shows the IHR of a 17-day-old embryo before and after administration of atropine. The *V* patterns that appeared frequently before administration of the drug were blocked by atropine, and the baseline HR was elevated. Figure 4B shows the IHR of a 14-day-old embryo that was treated with norepinephrine. Administration of norepinephrine resulted in a raised baseline HR only, without accompanying marked HRI patterns. Norepinephrine administration did not always raise the baseline HR, and it did not induce marked changes in the HRI patterns in four other older embryos. Figure 4C shows the IHR of a 17-day-old embryo before and after administration of isoprenaline. The rapid acceleration of HR (lambda pattern) occurred just after drug administration, and a raised baseline HR followed. However, four other embryos treated with isoprenaline failed to show marked changes in the HRI patterns. Figure 4D shows the IHR of a 16-day-old embryo that was given phentolamine. Soon after drug administration the transient fall of the baseline HR was accompanied by rapid decelerations of HR (*V* patterns). In another 17-day-old embryo the baseline HR dropped by ~70 beats/min soon after administration of phentolamine, and the HRI was augmented by spontaneous accelerated patterns. However, such changes in HRI did not always occur; IHR seemed not to be different before and after drug administration in six other embryos. Figure 4E shows the IHR of a 17-day-old embryo given propranolol. The baseline HR was depressed, and the marked, rapid accelerations of HR (avian omega patterns), which occurred intermittently during the 30-min control period, were not observed after drug administration, although the HRI characterized by accelerations and decelerations remained. Baseline HR was depressed in two other embryos. The *V* patterns were frequently observed in one embryo, but no marked HRI patterns occurred in the other embryo after depression of baseline HR. In the third embryo the marked changes in IHR as well as baseline depression did not occur after administration of propranolol.

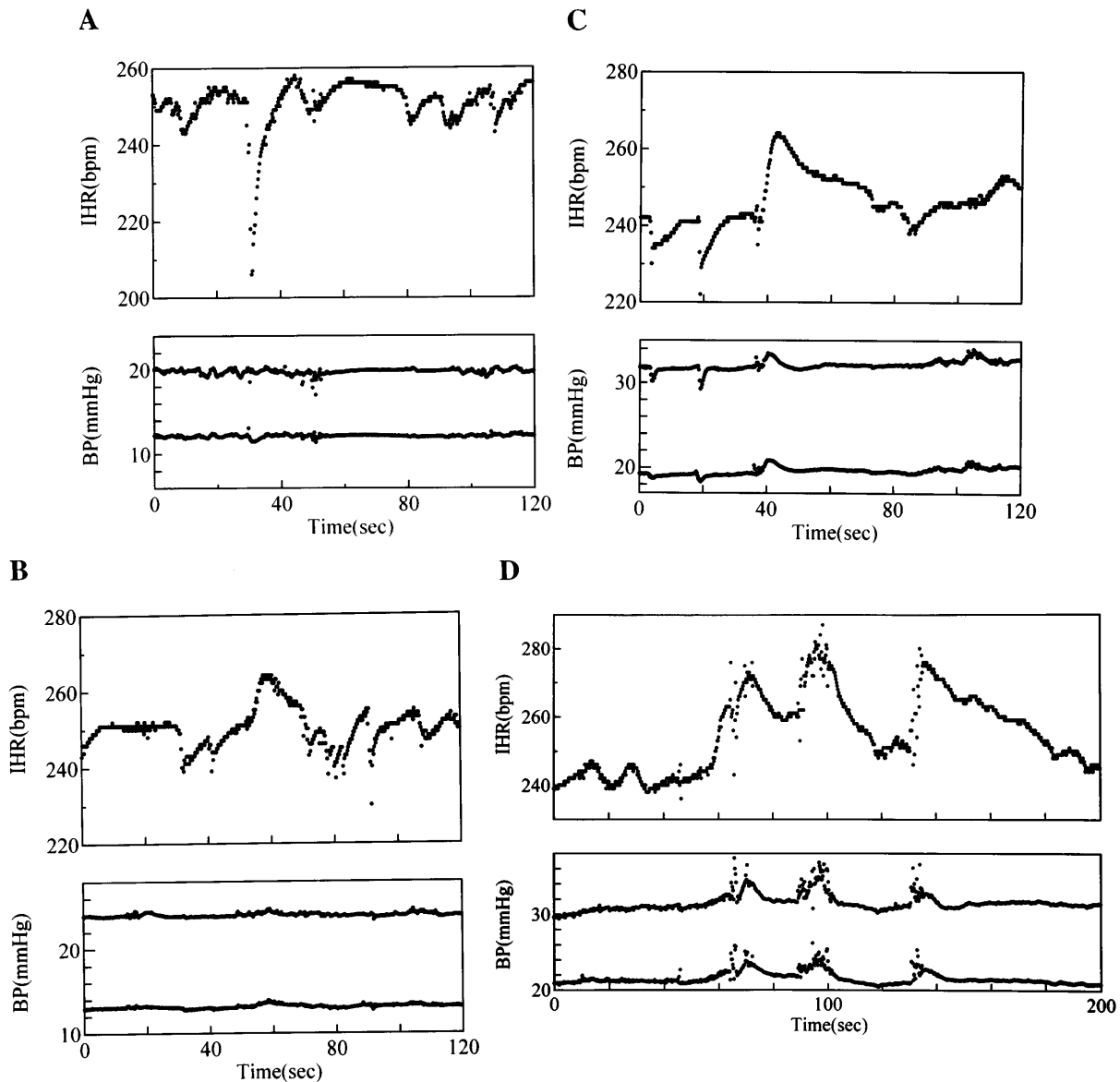


Fig. 2. Four patterns of heart rate (HR) irregularities (HRI) in IHR and systolic and diastolic blood pressures (BP). *A*: *V* pattern (★ in Fig. 1*C*) in 14-day-old embryo. Nadir of IHR was reached after 5 beats from baseline HR of ~250 to 205 beats/min. *B*: lambda pattern (● in Fig. 1*D*) in 17-day-old embryo. Although concurrent HR variability made baseline vague, baseline HR was determined to be 250 beats/min by averaging HR before and after HRI. *C*: avian omega pattern (○ in Fig. 1*E*) in 19-day-old embryo. Before and after this pattern, small *V* and lambda patterns appeared. Avian omega pattern was found from around *day 18* to hatching. *D*: periodic pattern (◆ in Fig. 1*F*) in 20-day-old embryo (same embryo as in *C*). To show baseline, recording is presented over a 200-s period. Three avian omega patterns occurred successively, and each pattern was associated with a small increase in BP. Scattered dots in BP and, accordingly, in HR plots seem to be artifacts, probably caused by embryonic movements. For BP patterns in *A–D*, top trace is systolic and bottom trace is diastolic BP.

## DISCUSSION

The avian eggshell plays an important role in the gas exchange of the embryo, which takes place by molecular diffusion through the pores between the external atmosphere and capillary blood of the CAM. In chicken eggs the CAM begins to spread under the inner shell membrane after the 1st wk of incubation and envelops the whole contents of the egg on around *day 12* of incubation. The CAM is well vascularized and serves as the respiratory organ for the prenatal embryo during the last 2 wk of incubation until the chick fetus

internally pips and then fractures the eggshell with its egg tooth (i.e., external pipping). The vascular bed of the CAM is supplied with blood by the allantoic artery, which forms a single stem as it leaves the embryo's body via the allantoic stalk. This stem divides into the right and left branches while passing on to the CAM. The diameter of these vessels was estimated at ~1–2 mm depending on the developmental stage of the embryo. Previously, the catheterization technique was developed for repeated and simultaneous removal of gas samples from the air cell and of blood from the

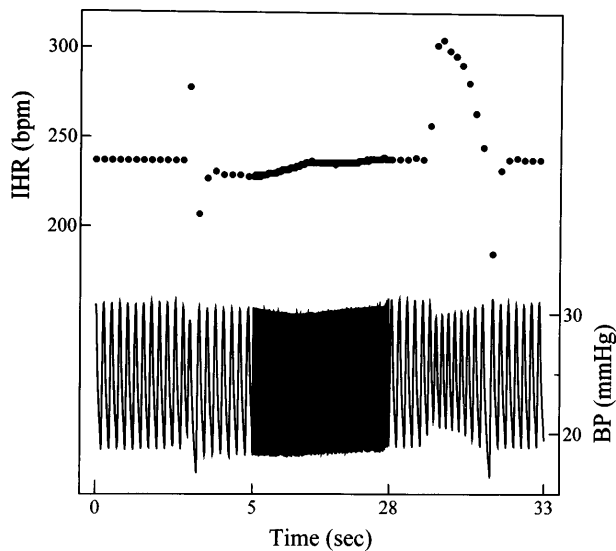


Fig. 3. A single-beat lambda pattern and a lambda pattern with single-beat deceleration that appeared during 33-s recording of IHR (top trace) and allantoic arterial BP (bottom trace).

allantoic blood vessels (18). Blood gas properties were not influenced by implantation of the catheter through a small hole opened in the eggshell and with subsequent closure of the hole. In other words, the catheter could be implanted while adequate gas exchange is maintained through the eggshell and CAM. This technique was developed to measure the blood pressure of late chick embryos (17), and we also used it to determine the IHR of chick embryos in the present study. Because of difficulty of catheterization after internal pipping, chick fetuses were not subjected to the blood pressure measurement.

The eggshell provides a unique advantage for noninvasive measurement of HR of the embryos developing within it. For instance, ballistic movements of the eggshell, which are attributed to cardiac contractions of the embryo (referred to as BCG), can be detected by various means, and the developmental patterns of embryonic HR in various species of birds have been investigated (5, 20, 22, 24, 25). Additionally, in association with cardiac contractions, acoustic pressure changes occur outside the eggshell; these changes can be detected by a condenser microphone attached to the eggshell with an airtight seal (referred to as ACG) (1, 14, 26). The ACG is less contaminated by the embryonic activity than the BCG. Taking advantage of the ACG, we measured noninvasively the IHR of chick embryos throughout the last half of incubation and found large HR fluctuations (i.e., HRI). Although the ACG method showed clearly the developmental patterns of HRI and has the advantage of noninvasive long-term measurement, the recording of IHR was sometimes contaminated by unknown artifacts (1). Therefore, we attempted to determine the IHR of chick embryos by measuring the blood pressure of the allantoic artery. The systolic and diastolic pressures were similar to those in previously cannulated eggs (16, 17, 19, 23). Mean HR were also similar to or a bit lower than those

reported previously (16, 17, 19, 23) and higher than those measured previously in fenestrated eggs (21).

When IHR determined from ACG was plotted against time, sudden accelerations and decelerations were shown (1). Similar outstanding points were reported in the human fetus and considered to be artifacts (27). However, blood pressure recording gives evidence that these single-beat alterations are true cardiac events (Fig. 3). The only possible noncardiovascular cause for blood pressure changes at the site of measurement was embryonic movements. In some cases, these movements caused occlusion of the allantoic artery, distorting the blood pressure waves. The distortion of the blood pressure waves produced artifacts in IHR such as these sudden changes in HR. However, as shown in Fig. 3, the pressure wave was not distorted, and sudden accelerations and decelerations indicate real HRI. These short-term irregular patterns were characterized by

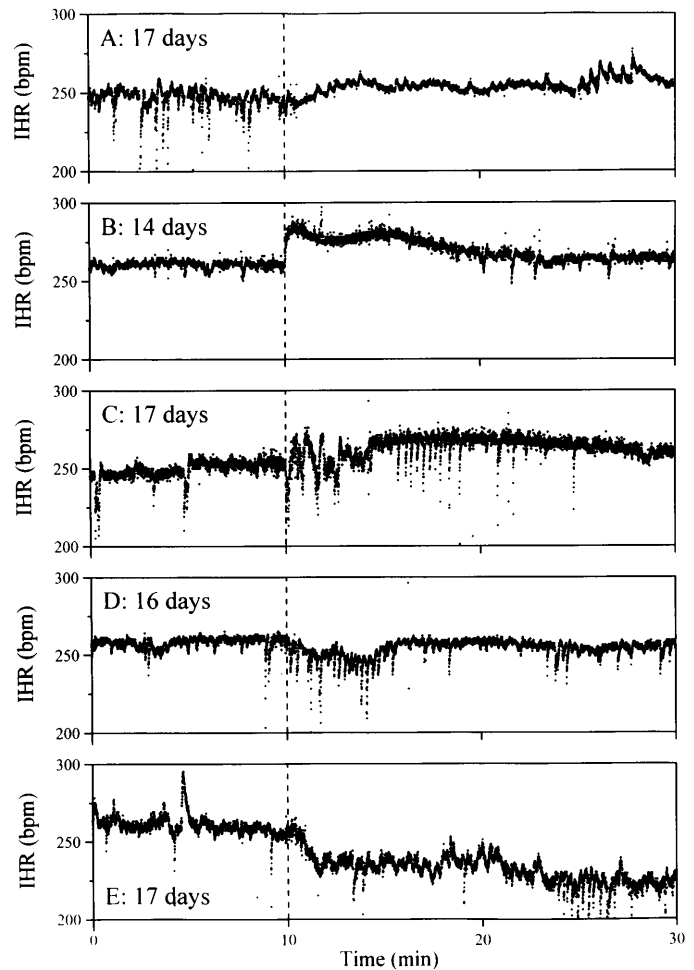


Fig. 4. Responses of IHR to administration of autonomic drugs. Although IHR was recorded for 30-min and 1-h periods before and after drug administration, respectively, a 30-min recording of IHR only is shown in A–E. Dashed line, time of drug administration. A: 5  $\mu$ g of atropine in 20  $\mu$ l of solution administered to 17-day-old embryo. B: 1  $\mu$ g of norepinephrine in 10  $\mu$ l of solution administered to 14-day-old embryo. C: 1  $\mu$ g of isoprenaline in 50  $\mu$ l of solution administered to 17-day-old embryo. D: 10  $\mu$ g of phentolamine in 10  $\mu$ l of solution administered to 16-day-old embryo. E: 1  $\mu$ g of propranolol in 20  $\mu$ l of solution administered to 17-day-old embryo.

shortened beat-to-beat intervals followed by a prolonged one. During the shortened beat-to-beat intervals, the diastolic pressure increased and the systolic pressure decreased in comparison with the regular beats (Fig. 3). It is likely that these short-term irregularities might be caused by an ectopic extrasystole without compensatory pause or ventricular automatism (e.g., ventricular flutter). These short-term HRI patterns were found before the baseline HR became irregular with appearances of augmented decelerated and accelerated patterns.

The HRI patterns of chick embryos described here were similar to some of the patterns previously reported for human fetuses. Hammacher et al. (6) described short-term bradycardias as dips. In their study, dips with  $\geq 1$ -min duration occurred in association with uterine contractions (*dips I* and *II*), whereas very short bradycardia occurred spontaneously, independently of labor (*dip 0*). In chick embryos, decelerations (bradycardias) of relatively long duration, referred to as *dips I* and *II* in human fetuses, were not found. Instead, the *V* pattern occurred within a very short duration, and the nadir was often reached within one or two heartbeats, which may correspond to *dip 0* in the fetus. This short-term deceleration pattern was also reported in the fetus by Wheeler and Murrills (28), who found a rapid decline in fetal HR, reaching the nadir within one to four beats and recovering to baseline over a longer period. In addition, Wheeler and Murrills reported decelerations and accelerations in human fetal heart rate that were similar to our findings in chick embryos. In humans, decelerations were typical of early recordings (21–27 wk of gestation) and were present in all recordings up to 29 wk of gestation. In contrast, the first acceleration patterns were noted at 25 wk of gestation (i.e., after  $\sim 60\%$  of total gestation time). As gestation advanced, they occurred frequently and were seen in all recordings by 34 wk. In chick embryos, decelerations were also the first to occur and were the dominant HRI patterns between *days 13* and *19* of incubation. Accelerations ( $\lambda$  and avian  $\omega$  patterns) began to appear on *day 15–16*, i.e., after  $\sim 70\%$  of total incubation time.

Human fetal HR has been studied during uterine contractions and fetal movements. It is interesting to note that the decelerations designated as *dips I* and *II* in human fetuses were absent in chick embryos, which are totally independent of maternal uterine functions. In addition, an elliptical pattern, which was reported to be a sustained rise in human fetal HR (2), was also absent in chick embryos. Aladjem et al. (2) reported that multiple human fetal movements caused elliptical and/or periodic patterns, isolated movements elicited the  $\omega$  pattern, and either type of movement elicited the  $\lambda$  pattern in human fetuses. The relation between human fetal HR and fetal movements was also reported by some other investigators (3, 15, 28). In avian eggs, embryonic movements can be easily measured by BCG recording, and thus further investigations into the relation between embryonic movements and HRI are feasible.

In chick embryos the development of innervation of the heart has been well studied. Adrenergic and cholinergic receptors in the heart are functional on about *day 4* of incubation (9). Parasympathetic innervation appears in the 1st wk of incubation (8), and sympathetic nerve fibers are present in the heart on *day 10* (11). On around *day 12* the parasympathetic innervation of the heart is functional, and on around *day 16* the sympathetic innervation is functional (7, 10). The present additional experiments with autonomic drug administration clearly demonstrated that the rapid, transient deceleration of HR (*V* pattern) was blocked by atropine (Fig. 4A). The administration of 2  $\mu\text{g}$  of ACh stopped the heartbeat during a short period of  $< 1$  min, and the HR decreased to zero with subsequent prompt recovery to the original baseline HR in five embryos tested. In two other embryos whose heartbeat was delayed by ACh, the HR reached a nadir within a few heartbeats, with subsequent recovery to the original baseline, resembling the *V* patterns. It is doubtless that the first occurrence of decelerated patterns of embryonic HR corresponds to functional initiation of the parasympathetic innervation, and the rapid, transient decelerations of HR (*V* patterns) are mediated by the parasympathetic nervous system. Meanwhile, the present additional experiments did not always show clear responses of HRI to administration of sympathomimetic and sympathetic blocking agents, which proved that the accelerated patterns of HRI were mediated by the sympathetic nervous system. However, inasmuch as the rapid decelerated patterns were mediated by parasympathetic nerves and in some embryos the accelerated patterns were observed but disappeared in others after the administration of sympathomimetic and sympathetic blocking agents (Fig. 4, *C* and *E*), it is likely that the accelerated HRI coincides with the initiation of sympathetic nervous function and the accelerated patterns are mediated by the sympathetic nervous system. Nevertheless, additional experiments with autonomic drug administration are required to fully investigate the development of sympathetic influences on chick HR control.

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