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Ontogenetic Development of Autonomic Neuroeffector Transmission and Transmitter Reactivity in Embryonic and Fetal Hearts*

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I. Introduction

The development of synaptic relationships between nerve and muscle cells has attracted much attention (51). Factors that contribute to the establishment of synaptic connections have been studied in the voluntary and autonomic divisions of the peripheral nervous system. Isolated neuromuscular preparations as well as cell culture techniques have been used to explore the roles of nerve cell and of muscle cell development in the establishment of synaptic connections.

The subject of this article is the development of autonomic neuromuscular relationships in the embryonic, fetal, and neonatal heart. Our understanding of the relationship between nerve and heart muscle cells has been enlarged by examining systems from adult animals in which the motor innervation of the muscle cell has been removed by surgical or pharmacological means. It is advantageous to examine the relationship under circumstances in which the neural elements begin to transmit information to muscle cells. It then becomes possible to study the properties of

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the muscle cell before and after innervation simply by examining the system during the course of ontogenetic development. Development of autonomic neuroeffector relationships will be analyzed in connection with the questions:

1. When do efferent autonomic nerves appear in the heart and when do the nerves contain transmitter?
2. When do autonomic nerves begin transmitting information to effector cells?
3. When do the receptors for transmitter agents appear?
4. What relationships exist between the development of neuroeffector transmission and the development of receptors for transmitter agents?

Although the answers to these questions are incomplete, several aspects of the development and establishment of cardiac neuromuscular relationships emerge.

This report will not consider the development of afferent autonomic pathways and cardiovascular reflexes; this is dealt with elsewhere (34, 40). In addition to these, the reader may wish to consult reviews that deal with the phylogenetic development of the autonmic nervous system (25), the autonomic innervation of the heart (138, 162, 163), the ontogenetic development of the cardiovascular system (7, 135), and the pharmacology of heart cells during development (119). Other publications that are helpful include the cardiovascular physiology of the bird (146) and the comparative development of cardiac cell properties *in vivo* and *in vitro* (97).

II. Synopsis of the Development of the Heart

The vertebrate heart develops from a tubular connnection between the extraembryonic and intraembryonic blood vessels. A helpful guide to the comparative development of cardiac muscle in seven vertebrates, including man, has been given by Sissman (141). The features of cardiac development to be described will be taken primarily from the chick embryo; the human heart (23) and chick heart display similar features during early development with the latter changing at a more rapid rate. The tubular chick heart begins contracting spontaneously at the 9-somite stage (33–38 hr after fertilization), shortly after myofibrillogenesis has been detected in ventricular myocytes (103). First contractions have been noted in mouse, rat, and human hearts at 8, 9, and 22 days after fertilization, respectively (141). The sporadic twitches of the chick heart are succeeded by regular pulsatile contractions at the 15-somite stage (about 48 hr after fertilization) and the tubular heart has assumed an "S" shape. The ventricles, atria, and sinus venosus can be identified morphologically as separate components of the cardiac tube at the 9-somite, 16-somite, and 25-somite stages, respectively. These three cardiac regions can be identified electrophysiologically at a time when no morphological separation is evident and when the heart has not begun to contract. Action potentials were recorded from sinoatrial and from ventricular cells at the 8-somite stage (28 hr); the sinoatrial action potential preceded the ventricular action potential (155). At an early stage of development, the dominant cardiac pacemaker is in the presumptive sinoatrial region and there is a characteristic delay between atrial and ventricular excitation. Action potentials characteristic of atrial, ventricular, and bulbar regions were recorded from presumptive cardiac areas explanted from chick blastoderm and allowed to develop *in vitro* (92). It is noteworthy that when the presumptive cardiac area is separated into anterior, middle, and posterior fragments, actions potentials resembling those of bulbar, ventricular, and atrial cells, respectively, were recorded. Therefore, the differentiation of presumptive cardiac cells into specific cell types occurs early in development.

Circulation of blood between extraembryonic and intraembryonic regions begins at the 16 to 17-somite stage (45–49 hr after fertilization). Moreover, the operation of the Frank-Starling mechanism has been

demonstrated as early as the 3rd incubation day *in vivo* (50) and the 4th incubation day *in vitro* (106). Enlargement and rotation of the cardiac tube occur rapidly. Septation is nearly complete in the atria by the end of the 5th incubation day and in the ventricles by the 6th incubation day. The pericardium develops as a sac-like structure around the heart between the 6th and 8th incubation days. The chick heart has the appearance of its adult counterpart by the end of the first week of life *in ovo*; hatching occurs 21 days after fertilization. At this time, the general development of the chick has reached a stage comparable to mouse, rat, and human fetuses at 15½, 17 to 18, and 47 days after fertilization, respectively. The gestation times for mouse, rat, and man are 19½ days, 22 days, and 40 weeks, respectively (141).

III. Ontogenesis of Parasympathetic Innervation of the Heart

A. Morphological and Chemical Studies

Vagus nerve fibers have been detected in the *truncus arteriosus* (aorta precursor) of the chick embryo heart between 64 and 68 hr. The possibility that these first vagal neurons were sensory in function (149) has never been tested. Elements of the vagus nerve were reported in the interatrial septum on the 5th incubation day (89), the sinoatrial region on the 6th incubation day, and in the atrial wall at the end of the 7th incubation day (1). Ganglion cells were detected between the 4th and 6th incubation days (148) and the bulbar (ventricular and aortic) and atrial plexuses were clearly established by the end of the first week of life in *in ovo*.

Zachs (164) noted that the chick embryo heart reacted positively to a histochemical test for the presence of cholinesterase at the 68 to 72-hr stage. Acetylcholinesterase was detected in the neural folds of the chick as early as the 1st incubation day. Since the detection of acetylcholinesterase preceded the morphological differentiation of nerve cells and the appearance of acetylcholine in the embryo, an inductive action of the enzyme on differentiation was considered (164). Gyévai (66) confirmed the observation that the appearance of acetylcholinesterase in cardiac muscle cells preceded innervation by the vagus nerve in both the chick and rat hearts. Innervation of the fetal rat heart by the vagus is thought to occur after the 13th gestational day. Cholinesterase-positive neurons and ganglia have been found in the ventricular myocardium of chicks and full-term mammalian fetuses of man, monkey, cattle, and sheep (53, 143). Nerve cells enter the human heart at the 15-mm stage (about 37 days after fertilization) and an epicardial plexus of several ganglia develops during the next 7 weeks (142). At the end of the embryonic period of development (8 weeks after ovulation), fibers from the right vagosympathetic trunk enter the sinoatrial node and interatrial septum while fibers from the left vagosympathetic trunk enter the aorta, pulmonary trunk and veins, and the left vena cava (56). The presence of ganglia in the human heart was confirmed in this study (no thoracic sympathetic fibers to the heart were found at this stage). The ganglia in the human embryonic heart (56, 142, 143) may be the cell bodies of afferent sensory neurons or of efferent postganglionic neurons. Neither cholinesterase staining nor perikaryon shape (multipolar *vs.* bipolar or unipolar) has conclusively identified postganglionic cholinergic nerves in the vertebrate heart.

Acetylcholinesterase has been found in association with rough surfaced endoplasmic reticulum of the fetal rabbit cardiac myocyte on the 9th gestational day (68). The appearance of the enzymatic reaction product in the sarcoplasmic reticulum later in development supported the view that the membrane of the sarcoplasmic reticulum is derived from the rough endoplasmic reticulum. As in other vertebrates studied, acetylcholinesterase appeared in fetal rabbit cardiac muscle cells before innervation occurred. The view that cholinesterase is involved in differentiation of

cardiac muscle cells and in the appearance of automaticity has often been suggested (84). However, these proposals have not been tested in developing animals.

Considerably less information is available concerning choline acetyltransferase and acetylcholine in the embryo heart. An increase of choline acetyltransferase activity occurred on the 10th incubation day in chick embryo heart; the activity of this enzyme continued to increase throughout the remaining period of life *in ovo* (62). The results of this study have been confirmed and refined by means of more sensitive chemical measurements (134). Both studies agree that a neuronal source of choline acetyltransferase is detectable at the 10th to 13th incubation day stage. However, Gifford *et al.* (62) concluded that there was an extraneuronal store (heart muscle) of choline acetyltransferase in the young (4th to 9th incubation day) chick embryo heart. This observation is disputed by Roskoski *et al.* (134) who did not detect the enzyme with a sensitive and specific chemical assay; they concluded that the enzyme is absent from heart muscle cells. Measurements of choline acetyltransferase activity do not indicate the origin of the enzyme since it could be located either in preganglionic or in postganglionic cholinergic fibers of the efferent vagus. Furthermore, experiments have yet to determine when vagal neurons are capable of synthesizing acetylcholine from choline and acetyl coenzyme A. High affinity choline uptake is said to be a sensitive indicator of cholinergic neuron development (144); measurements of choline transport could assist in determining the development of intracardiac vagal neurons.

Measurable amounts of an acetylcholine-like substance have been detected in the chick embryo heart (99) placed in organ culture. The detection of an acetycholine-like material in these experiments was associated with the histological demonstration (silver stain) of nerves within the heart. Although no nerves were detected in embryo hearts at 48 hr, neurons were seen at 60 hr after fertilization—several hours earlier than the time described by others. The hearts were placed in organ culture along with segments of spinal cord; the latter were removed several days before biological assay for the acetylcholine-like material. Cardiac content of the acetylcholine-like material and of intramural nerves increased in parallel in these organ culture experiments. However, acetylcholine content and disposition have not been measured in the heart isolated from embryos of different ages. In addition, the ultrastructural appearance of developing cholinergic nerves in the heart has not been described.

B. Cholinergic Neuroeffector Transmission

Inhibitory transmission between postganglionic neurons and sinoatrial pacemaker cells was detected for the first time on the 12th incubation day in the isolated chick embryo heart (121). Inhibition was cholinergic in nature because inhibitory transmission was associated with membrane hyperpolarization due to an increased conductance to K^+ and was blocked by atropine, as in adult vertebrate hearts (152). The inhibitory effect of field stimulation was also blocked by tetrodotoxin at a concentration that did not significantly change pacemaker activity. This finding pointed to a neural origin of the inhibitory effect. Detection of inhibitory cholinergic transmission occurred on the 10th incubation day in the presence of physostigmine in the chick embryo heart. The effect of physostigmine was attributed to inhibition of cholinesterase which allowed small amounts of released acetylcholine to reach sensitized postsynaptic receptors (101) in concentrations sufficient to inhibit pacemaker activity. Evidence that the appearance of cholinergic inhibitory transmission depended upon the presence of sufficient amounts of transmitter available for release was provided by experiments done with nicotine and dimethylphenylpiperazinium (118) (see section III C 2).

These data do not agree with the conclusions drawn from experiments done with

chick embryos at 4 to 6 days incubation age (93). Injection of acetylcholine into the region of the 4th ventricle (just caudal to the auditory vesicle level) caused inhibition of the heart in 5-day (120–126 hr) and 6-day (144–150 hr) but not in 4-day (96–101 hr) chick embryos. It was concluded that the efferent (motor) vagal pathway to the heart began to function at the 5th incubation day. This interpretation assumed that at least two synapses (ganglion and neuroeffector) were functioning on the 5th incubation day. However, this assumption was not tested with pharmacological antagonists such as atropine, hexamethonium, and tetrodotoxin. Therefore, it is uncertain that a vagal cardioinhibitory mechanism functioned on the 5th incubation day.

The results of electrophysiological and pharmacological studies of the development of cholinergic inhibitory transmission to the sinoatrial pacemaker are in accordance with the view that the amount of transmitter available for release is responsible, in part, for determining the onset of transmission. However, the amount of transmitter present in the heart at different stages during ontogenesis is not known. In addition, the presence of acetylcholine in a neuronal component derived from the heart should be determined and compared to the onset of transmission.

The reason for underscoring the cellular location of the acetylcholine is shown in the work of Coraboeuf *et al.* (36). They reported that the chick embryo heart contained an acetylcholine-like material in the non-innervated state. Electrical stimulation of the heart at frequencies greater than normal evoked an inhibition of automaticity that occurred at the end of the stimulus period. It seemed that the inhibition was not the result of overdrive (156) since it did not occur in the presence of atropine. The inhibitory effect was augmented by elevation of the concentration of K^+ or Ca^{++} in the bathing fluid, procedures that can increase the release of transmitter agents from nerve cells (85). An acetylcholine-like material was detected when the pooled effluent from stimulated hearts was assayed on leech muscle. Coraboeuf *et al.* (37) proposed that the acetylcholine-like material originated either in cardiac muscle cells or in connective cells. The results of this study are intriguing for they could be viewed in accordance with the proposal that acetylcholine, released from heart cells, could act upon heart cells through a negative feedback mechanism and regulate automaticity. It remains to be demonstrated that the intracellular organelles (multivesicular bodies and atrial granules) described by Coraboeuf *et al.* (37) actually contain and release transmitter agents. Multivesicular bodies in heart muscle cells contain 500 Å vesicles that resemble those found in other cell types (108); it has been proposed that multivesicular bodies in the heart, as in other cells, have lysosomal activity (108).

The chick atrium (10) and ventricle (18) have an inhibitory cholinergic nerve supply. Stimulation of intracardiac nerves evoked a negative inotropic effect that was followed by a positive inotropic effect. The negative inotropic effect of field stimulation was prevented by atropine or hyoscine but not by the ganglion blocking agents, hexamethonium or pempidine. The pharmacological properties of inhibitory transmission to the sinoatrial pacemaker, atrial muscle, and ventricular muscle cells are quite similar. That is to say, field stimulation initiated impulses in postganglionic neurons and released acetylcholine that evoked characteristic inhibitory effects on nonpacemaker and pacemaker cells. Blockade of the inhibitory effect of field stimulation in the ventricle by triethylcholine was attributed to impairment of acetylcholine synthesis, a mechanism previously described at the skeletal neuromuscular junction (22). There are no data concerning the onset of cholinergic neuroeffector transmission in either the avian atrium or ventricle. Inhibitory innervation of atrial muscle may not occur at the same time as that of the sinoatrial pacemaker. Experiments that examined this problem would allow one to determine the

developmental pattern for functional innervation of the upper heart by inhibitory vagal neurons. In addition, it is not known whether the negative inotropic effect mediated by cholinergic receptors precedes the appearance of inhibitory cholinergic innervation of the avian ventricle. Since acetylcholine had inhibitory effects on ventricles from 3-week-old chicks (18) and had no inhibitory actions on ventricles up to the 19th incubation day (140), it seems that the cholinergic receptors causing inhibition develop much later in the ventricle than in the atrium.

Cholinergic innervation of ventricular muscle from birds seems to be rather well developed when compared with that from atrial muscle with respect to density of terminal nerve fibers and intensity of effect. In mammalian hearts the ability of the parasympathetic nervous system to reduce contractility of ventricular muscle is considerably less than that observed in atrial muscle (71). It is noteworthy that stimulation of vagal fibers to the isolated, perfused heart of the adult chicken released large amounts of acetylcholine (about 100 to 250 pmole g^{-1}/min^{-1} in the absence of cholinesterase inhibitors (45, 86). The ventricular muscle content of acetylcholine and of cholinesterase is higher in the chicken than in the rabbit and both the innervation by cholinergic fibers and sensitivity to applied acetylcholine are greater in the chicken than in the rabbit (86) or in the cat (87). The ability to recover large amounts of acetylcholine in the perfusate in the presence of large amounts of cholinesterase may be related to the cytological disposition of the transmitter and enzyme (86).

There is little systematic information regarding the onset and development of cholinergic inhibitory neuroeffector transmission in mammals with the exception of man. Field stimulation inhibited contractions in atria taken from human fetuses at the 8- to 9.9-mm (crown-rump length) stage but not before this stage (158). The negative inotropic effect of field stimulation reached a maximum in 5 sec, was augmented by neostigmine, and was blocked by atropine or tetrodotoxin. These findings are qualitatively similar to those obtained in the chick embryo heart. The similarity extends to the observation that the ontogenetic appearance of the inhibitory effects of nerve stimulation precedes that of the excitatory effects (see above).

Stimulation of the cervical (preganglionic) vagus nerve evoked a small (10%) inhibition of the fetal lamb heart as early as the 60th gestation day; the cardioinhibitory effect increased with age during fetal and postnatal life (21). However, experiments have not been done to determine the ontogenetic appearance of cholinergic neuroeffector transmission in the lamb. Efferent vagal (preganglionic) stimulation slowed the sinoatrial pacemaker in newborn rabbits (41, 139) whereas no reduction in heart rate occurred when epinephrine was given to fetal rabbits (41). The failure of epinephrine to inhibit the fetal rabbit heart reflexly could have been due to the absence of afferent or central cardiovascular reflex components as well as to a nonfunctional efferent pathway. The inhibitory effect of vagal stimulation was enhanced by physostigmine, particularly in young (1 week) rabbits in which the inhibitory effect had been depressed by stimulation at high ($\geq$ 50 Hz) frequencies (139). The depressant effect of high frequency stimulation, which was attributed to depolarization block by virtue of intraneuronal Na^+ accumulation, was not readily evoked in older animals. It is important to note that the inhibitory effects of vagal stimulation were partially opposed by the type and concentration of anesthetic used. Sodium pentobarbital reversibly diminished the inhibitory effect of vagal stimulation; newborn rabbits were more sensitive to this action of anesthetics than were 6-week-old rabbits. This finding illustrates the difficulties encountered in determining the properties of neuroeffector junctions in preparations exposed to anesthetic agents.

C. Reactivity to Cholinergic Agonists and Antagonists

1. Muscarinic drugs. The interaction between cholinergic drugs and the developing heart will be considered with regard to the onset of drug action during ontogenesis, the change in reactivity with particular reference to innervation, and the mechanism of action. John Pickering (125), in 1893 considered all of these aspects in his paper dealing with the physiology of the embryonic heart, published over 80 years ago. The results of Pickering's experiments in 1896 (127) with electrically-induced alterations in cardiac rhythm convinced him that the chick embryo heart did not respond to muscarine until 200 hr incubation, at which time inhibitory innervation of the organ was demonstrable (126), he concluded. Similarly, atropine was found to be without specific effect on the rate of spontaneous beating in the chick embryo heart although it was capable of producing a nonspecific depression of automaticity. [This observation has been recently described by Coraboeuf *et al.* (36)].

Subsequent investigations showed that the inhibitory effects of acetylcholine on the sinoatrial pacemaker in the chick heart could be demonstrated earlier than reported by Pickering with muscarine. Platnner and Hou (128) concluded that the inhibitory effect of acetylcholine did not require innervation since it was observed at 72 to 94 hr after fertilization. Indeed, acetylocholine evoked an atropine-sensitive inhibition of the chick heart pacemaker at the 10-somite stage or 33- to 38-hr embryo (38, 74). Therefore, the receptor for acetylcholine had been synthetized and the coupling of the receptor to the inhibitory mechanism had been accomplished in the chick embryo heart membrane at a time when spontaneous contractions are first detected. Hsu (74), as well as Cullis and Lucas (38), reported that "tolerance" to the inhibitory effect of acetylcholine developed during continuous exposure to the drug and that a second exposure to acetylcholine had less inhibitory effect even though the heart had been washed in saline solution between exposures. Furthermore, the tolerance to acetylcholine was not due to hydrolysis of the drug by cholinesterase since recovery from inhibition occurred even in the presence of physostigmine, an observation confirmed by Obrecht-Coutris and Coraboeuf (111).

In 1960, Dufour and Posternak (47) confirmed the early ontogenetic appearance and the brief duration ("adaptation") of the inhibitory effects of acetylcholine in the chick embryo heart. They also found that the sensitivity of the atria to acetylcholine increased between the 2nd and 3rd incubation days on the one hand and the 7th to 10th incubation days on the other hand. [Acetylcholine also inhibited automaticity in isolated ventricular muscle segments, particularly at the base. The frequency of beating of the apex, which was slower than that of the base, was often accelerated by high concentrations (10^{-4} to 10^{-3} g/ml) of acetylcholine.] Dufour and Posternak (47) concluded that it was not possible to attribute the increased acetylcholine sensitivity to innervation which was assumed to occur at the 5th to 6th incubation days.

Studies done in the developing rat heart noted that cardiac pacemaker sensitivity to acetylcholine increased with age (69, 113). However, the results of these investigations differ with respect to the relationship between reactivity to acetylcholine and cardiac innervation. Hall (69) found that acetylcholine inhibited the rat heart at the 11½-day stage and concluded that this response preceded vagal innervation of the heart. On the other hand, Pager *et al.* (113) reported that acetylcholine had little or no effect on day 13 whereas its inhibitory action was present by day 16, that is, after morphological innervation had occurred. It is possible that the lower temperature (24°C) used by Pager *et al.* (113), which reduced basal pacemaker frequency, impaired the detection of acetyl-

choline-induced inhibition. The importance of temperature has been emphasized by Robkin *et al.* (131) who conducted experiments with explanted rat embryos at 37°C and confirmed the observations made by Hall (69). Results obtained in experiments done with fetal mouse hearts are similar to those described by Hall (69) for the rat. Wildenthal (160) reported that the responsiveness of the fetal mouse heart to acetylcholine increased steadily with age from the 13th day to the time of birth (21–22 days). It is assumed that parasympathetic fibers enter the mouse atrium on the 14th to 15th gestation day and that innervation increased in density thereafter (160). Therefore, the mouse heart sinoatrial pacemaker has receptors that interact with acetylcholine before vagal innervation has occurred. It would be of interest to determine whether, as in the chick heart, a temporal relationship exists between the functional innervation of the mouse heart and altered sensitivity to acetylcholine (see below).

There are at least two examples that do not follow the pattern of increased reactivity to acetylcholine with age. The sensitivity to inhibition by acetylcholine was the same in the mature fetal lamb and in the adult (53). However, the reaction to acetylcholine differed in fetal and adult sheep hearts as indicated by experiments with atropine. Opposition to the inhibitory effect of acetylcholine by atropine in the adult heart may have been due not only to antagonism of muscarinic inhibition but also to allowing the sympathomimetic effects of high concentrations of acetylcholine. The latter action was thought to be minimal or absent in the fetal lamb because of the incomplete development of the sympathetic nervous system (53). The ED50 for the inhibitory effect of acetylcholine on the sinoatrial pacemaker increased with age from newborn to adult stages in the rabbit (25). The greater sensitivity of the heart of the newborn rabbit to the negative chronotropic effect of acetylcholine was attributed to a lower acetylcholinesterase content that permitted greater preservation of applied acetylcholine.

It had been concluded that innervation by the vagus, which was thought to occur on the 5th to 6th incubation day, had no effect on reactivity of the chick embryo heart to acetylcholine (52, 106). It is of interest that Fingl *et al.* (52), who recorded intracellular action potentials in chick embryo hearts from the 3rd to the 7th incubation day, noted that functional innervation of the heart may not have taken place during this period of time when histological evidence suggested that morphological innervation had occurred. The experiments done in Shideman's laboratory (106) also showed that the ability of physostigmine to augment the inhibitory effect of exogenously applied acetylcholine was absent in 3-day hearts, appeared in 4-day hearts, and increased in 8-day hearts. This phenomenon has been reexamined (94) and it was found that the inhibition by acetylcholine had the same concentration-effect relationship and the same reactivity to atropine in 3- and 4-day chick embryo hearts. However, physostigmine augmented the inhibition by acetylcholine only in 4-day preparations, an effect attributed to an increase in the activity of acetylcholinesterase (94). In view of the observation by Zachs (164) that cholinesterase was demonstrable histochemically in the heart of 3-day chicks, it is possible that the onset of the ability of physostigmine to prolong the effect of acetylcholine is due to location as well as activity of the enzyme. The duration of the inhibitory effect of acetylcholine was briefer in 4-day than in 3-day hearts in accordance with the proposal that hydrolysis of the drug by cholinesterase was an important feature in preparations from the 4th incubation day. These results differ from those obtained in other laboratories; the the inhibitory effect of acetylcholine is brief, even in the continuous presence of the drug, in hearts from the 10-somite to 10th incubation day stages (38, 47, 111, 122).

The sensitivity of the chick embryo

heart pacemaker to acetylcholine increased at about the time that cholinergic neuroeffector transmission could be demonstrated (101). The increased sensitivity to acetylcholine occurred on the 10th incubation day when transmission could be demonstrated only in the presence of physostigmine. Therefore, it was concluded that the last step in the onset of cholinergic transmission was related to an increase in the amount of transmitter available for release. The inhibitory effect of acetylcholine, which was brief before the onset of cholinergic transmission, increased in duration after this time. This phenomenon, which had been termed tolerance (38) and adaptation (47), was attributed to desensitization (122). Desensitization seemed to be specific, that is, the phenomenon occurred with addition of choline esters (acetylcholine, acetyl-β-methylcholine, and carbamylcholine) but not with an increase in the external concentration of K^+. Desensitization to choline esters was not as readily produced in sinoatrial preparations taken from animals at and after the onset of cholinergic transmission. Since the membrane conductance to K^+ apparently increased in atrial cells (116), it is possible that the increased duration of the inhibitory effect was due to a persistent increase in membrane potassium conductance in cells from the older preparations. During ontogenesis there is an increase of potassium permeability and conductance of atrial and ventricular muscle cells in chick and rat hearts (14, 28, 116, 145). Desensitization might also be related to a change in the properties of the receptor; determination of the chemical properties of the receptor would help the analysis of this problem. [The possibility that muscarinic cholinergic receptors can be detected in the developing chick heart has been tested by exposing the tissue to the reversible antagonist, 3-quinuclidinyl benzylate (136). The specific activity of tritium-labeled antagonist bound to components in the microsomal fraction increased from the 3rd incubation day to the day of hatching; specific activity decreased slightly in adult hearts. Experiments that compare receptor affinity for agonists and antagonists (chemically and biologically) are needed before the specific binding sites can be regarded as receptor sites. In addition, it will be necessary to determine the distribution (atrium *vs.* ventricle; intracardiac nerves *vs.* cardiac *vs.* vascular smooth muscle) of specific bindings sites and compare the results with the distribution of drug receptor sites in the developing heart.]

A. MECHANISM OF INHIBITION. The effects of acetylcholine and carbamylcholine have been studied on the electrical properties of sinoatrial cells in the chick embryo heart (36, 37, 52, 116). Pacemaker inhibition was accompanied by a decrease in maximum diastolic potential of sinoatrial cells on the 3rd and 4th incubation days (52, 116) and an increase in maximum diastolic potential after the 5th incubation day. Since removal of Na^+ from the bathing solution allowed drug-induced hyperpolarization of the pacemaker membrane, it was concluded that the choline esters increased Na^+ permeability (decreased maximum diastolic potential) and increased K^+ permeability (pacemaker inhibition and increased maximum diastolic potential) in the 3- and 4-day hearts. The ability of acetylcholine to increase membrane conductance to K^+ and to accelerate repolarization of atrial membranes and thereby reduce action potential duration increased progressively during development of the rat (113) and chick heart (116). Acetylcholine had no effect on atrial action potential duration in the 4-day chick embryo heart (36, 37, 116). This observation, which confirmed that made by Fingl *et al.* (52), supported the conclusion that the ionic mechanism underlying the membrane actions of acetylcholine changed during development. Coltart *et al.* (32) reported some puzzling results of experiments done with atria isolated from 12- to 13-week human fetal hearts (32). Carbamylcholine (10^{-5} g/ml) reduced twitch tension by about 45%; however, no change in

action potential duration was detected. Furthermore, carbamylcholine had no effect on automaticity when force was diminished. Usually, cholinomimetic drugs reduce twitch tension and atrial action potential duration simultaneously; automaticity is also inhibited by drug concentrations that have a negative inotropic effect (152). No mechanism has been advanced to explain the results obtained by Coltart *et al.* (32). It should be noted that some of the results reported by Coltart *et al.* (32) have not been obtained by others. Acetylcholine inhibited the sinoatrial pacemaker in human fetal hearts as early as the 10th week of life (59, 158). The discrepancy has not been resolved.

Acetylcholine inhibits the pacemaker in the adult vertebrate heart by increasing membrane conductance to K^+ (152). This mechanism also explains the inhibitory effect of acetylcholine in the developing rat (113) and chick heart (116). It is of interest that the ability of elevated external K^+ to suppress automaticity increases with develomental age (43, 116, 145) and this is paralleled by an augmentation of the ability of acetylcholine to reduce the action potential duration in the fetal rat heart (113) and in the chick embryo heart (116). The mechanism of the negative inotropic effect of acetylcholine in embryonic heart muscle could be due to a reduction in the intensity and duration of the depolarizing stimulus (action potential) as observed in atrial muscle of adult vertebrates. Voltage clamp experiments (63, 150) suggest that the negative inotropic effect of acetylcholine is due to actions of the drug on two ionic currents, the slow inward current (carried by Na^+/Ca^{++}) and a time-independent outward current (presumably carried by K^+). In the mammalian atrium (150) low concentrations of acetylcholine increased a steady state outward (presumably K^+) current that prevented the slow inward current (carried in part by Ca^{++}) from running its customary time course. At high concentrations, acetylcholine decreased the inward current and increased further the steady state outward current. In the amphibian atrium (63), contrasting results were obtained, for acetylcholine decreased the slow inward current at concentrations that did not increase the steady state outward current. If these different results were due to phylogenetic rather than to technical variations in experimental conditions, it would be desirable to examine the ionic basis of the negative inotropic effect of acetylcholine in avian cardiac muscle.

It is generally agreed that the inhibitory effect of acetylcholine increased with developmental age in avian and mammalian hearts. Whether the increased sensitivity is dependent upon innervation has not been resolved. The results obtained in the chick embryo heart indicate that there is a temporal relationship between the increased sensitivity of the pacemaker to cholinergic drugs and the appearance of cholinergic neuroeffector transmission. However, experiments that examine the relationship between interruption of the development of the postganglionic cholinergic nerve and sensitivity to cholinergic agonists are required to determine whether the relationship is causal. Experiments with chronic ganglionic blockade may be helpful in resolving this problem provided there is transsynaptic regulation of synthetic enzyme activity and transmitter concentrations in cholinergic neurons as there is in adrenergic neurons (15).

2. Nicotinic drugs. Nicotine and dimethylphenylpiperazinium, which release transmitter from autonomic nerves through mechanisms similar to those caused by the nerve impulse (157), began to inhibit the chick embryo heart pacemaker at about the time that cardioinhibitory nerve transmission began (118). It was concluded that nicotinic drugs stimulated hexamethonium-sensitive cholinergic postganglionic cells or axons and initiated tetrodotoxinsensitive impulses that released acetylcholine to inhibit pacemaker activity. Tetrodotoxin prevented the neurally-dependent inhibition of the

sinoatrial pacemaker caused by nicotine and dimethylphenylpiperazinium in the perfused sinus node artery preparation of adult dogs (30). High concentrations of nicotine (10^{-3} M) inhibited the pacemaker before functional cholinergic innervation; this was attributed to a direct action on heart cells because it was not prevented by atropine. These results differ from those of Lee *et al.* (91) who reported that nicotine (1.2×10^{-4} M) had a biphasic effect on heart rate (an increase followed by a decrease) in 4-day chick embryos. The decrease in heart rate became greater with higher concentrations of nicotine; however, the mechanism of the negative chronotropic effect was not described (91). It was not reported that the force of cardiac contractions changed with nicotine in untreated preparations; in the presence of atropine, nicotine increased the force of contraction.

Nicotine and dimethylphenylpiperazinium did not diminish the force of contraction in ventricular muscle isolated from chicks (at least 3 weeks after hatching). Since acetylcholine depressed the force of contraction in such preparations, it was concluded that pharmacological evidence for ganglion cells in the avian ventricle was lacking (18). Anatomical evidence [Hsieh (1951), cited in 18] indicated that vagal ganglion cells are present in the anterior cardiac (bulbar) plexus and that the postganglionic cholinergic fibers in the ventricle originate in this plexus. This conclusion was apparently supported by the observation that tetramethylammonium, 4-(m-chlorophenylcarbamoyloxy)2-butynyl trimethylammonium (McN-A-343), and 3-acetoxy-1-benzyl-1-methyl pyrrolidinium (AHR-602) evoked atropine-sensitive depression of ventricular contractions. However, the origin of the muscarinic action of these compounds (*via* cholinergic nerve stimulation or direct action on muscle cells) is not known. [Parenthetically, it is noteworthy that acetylcholine, nicotine, dimethylphenylpiperazinium, and AHR-602 had positive inotropic effects in the atropine-treated chick ventricle. However, tetramethylammonium did not increase the force of contraction in ventricular muscle (18) in contrast to the results obtained by Lee *et al.* (91) in 4-day to 12-day embryos. Although this can be the result of an age-dependent change in reactivity to tetramethylammonium, it is not easily understood in view of the persistence of sympathomimetic effects of acetylcholine and nicotine.]

IV. Ontogenesis of Sympathetic Innervation of the Heart

A. Morphological and Chemical Studies

The appearance of sympathetic nerve elements in the chick embryo heart is delayed with respect to the appearance of parasympathetic nerve elements (133). Sympathetic neuroblasts form the primary sympathetic chain about the 4th incubation day; a secondary sympathetic neuroblast migration that develops into sequential ganglia appears on the 6th incubation day and is well developed by the 8th incubation day. A branch from the superior cervical ganglion directed posteriorly toward the heart appears and reaches the heart at the 10th incubation day (reviewed in 133). The development of bulbar (aorta and ventricles) and atrioventricular (atrial) plexuses, which give rise to nerve fibers entering the heart, can be detected on the 7th incubation day in the chick embryo heart. As with the parasympathetic division, there is some uncertainty about the time of innervation of specific regions of the heart and about the development of definitive neuromuscular relationships.

Earlier studies of the sympathetic nervous system were summarized by Wechsler and Schmekel (159) who examined the ultrastructure of sympathetic and spinal ganglion cells in the chick embryo. Sympathoblasts could be distinguished from cortical cells in sympathetic ganglia by the presence of osmiophilic granules (60 to 170 nm) in the cytoplasm of the perikaryon and primary cell processes as early as 4.5

days after fertilization (159). The osmiophilic granules, or dense core vesicles, were also detected in the perikaryon of the 9-day-old embryo, but they are lacking in the perikaryon of the sympathetic ganglion cells of 3-week-old chicks. Dense core vesicles are found in synaptic regions of sympathetic ganglia in the same neuron (preganglionic) as are found agranular vesicles. Transport of the large dense core vesicles from the perikaryon to nerve endings can explain this finding. Agranular vesicles (40 nm) are characteristic organelles of cholinergic nerves and are thought to be storage sites for acetylcholine (see 8). Wechsler and Schmekel (159) pointed out that the large dense core vesicles had not been conclusively identified as storage sites for catecholamines. Large dense core vesicles have been found in both cholinergic and adrenergic nerves (8). (Although the large dense core vesicles can conceivably function as intracellular transport mechanisms for catecholamines, they may not be an inclusion characteristic of adrenergic neurons.)

Catecholamines were first detected by the Falck-Hillarp fluorescent histochemical method in the primary sympathetic chain at 3.5 days after fertilization (48). The intensity of the green fluorescence, characteristic of catecholamines, increases with age and is quite prominent by the 6- to 8-day stage when the secondary sympathetic trunk is formed. The appearance of a fluorescent reaction product characteristic of catecholamines occurs within 1 day of the detection of large dense core vesicles in the primary sympathetic trunk (159). Green fluorescent nerve fibers were found in the sinoatrial pacemaker region and in the ventricular musculature on the 12th (117, 120) and 16th (48) incubation days, respectively. Fluorescent fibers in the sinoatrial region first appeared in a large nerve trunk of the atrial plexus; varicose terminal fibers were not detected in the pacemaker region until the 17th incubation day. This is 1 day after fluorescent fibers were found in the ventricular musculature (48). The appearance of varicose nerve fibers has been associated morphologically with the establishment of neuromuscular relationships in the autonomic nervous system (8, 27). In chick hearts from 1 day after hatching through 24 weeks later, the density of fluorescent fibers was (in descending order): sinoatrial node, atrioventricular node, atrial musculature, and ventricular musculature (9). It was concluded that the dominant catecholamine in avian adrenergic nerves is norepinephrine and that most of the large amounts of epinephrine found in the heart (see below) were located in extraneuronal stores. However, the paucity of fluorescent extraneuronal structures pointed to a discrepancy between biochemical and histochemical findings.

The ontogenetic appearance of catecholamines within the heart has been studied in several laboratories (see 110). One of the most complete studies has been done on the chick embryo heart by Ignarro and Shideman (76–79). It was concluded that norepinephrine and epinephrine moved from the yolk to the chick embryo and heart on the 3rd to 4th incubation day (78) and that the heart was not capable of synthetizing these catecholamines at this stage of development (77). The concentrations of norepinephrine and epinephrine remained low from the 3rd to the 7th incubation days when a rise occurred that reached a peak by the 10th incubation day (77). Large variations in the cardiac content of these catecholamines occurred after this time and up to 42 days after hatching. Qualitatively similar variations in catecholamine content of the chick heart were reported by Manukhin *et al.* (104). The ratio of norepinephrine to epinephrine was greater than one from the day of hatching until 6 days later, less than one from 6 days until 28 days after hatching, and greater than one up to 6 months after hatching (77). This was confirmed by Manukhin *et al.* (104); however, they also found that the ratio of norepinephrine to epinephrine was greater than one

throughout embryonic life in contrast to the findings of Ignarro and Shideman (77). The results of both of these studies showed that the concentrations of norepinephrine and epinephrine in the chick heart underwent cyclic variations that were quite marked during embryogenesis and after hatching.

Endogenous norepinephrine content in the neonatal rat heart was only 5% of adult levels at birth and had reached 80% of adult levels 3 weeks after birth (81). Adult levels, measured at 42 days after birth, averaged 0.95 ± 0.07 μg of norepinephrine per g of heart. [In contrast, the content of endogenous norepinephrine and epinephrine in the chick heart decreased after hatching (77).] The ability of the rat heart to accumulate ^{3}H-norepinephrine developed in parallel with the endogenous norepinephrine content. The retention of ^{3}H-norepinephrine by the rat heart was small in rats from 1 to 22 days old; retention attained adult levels by 6 weeks after birth (65). It was assumed that the norepinephrine content reflected the density of sympathetic innervation of the heart by virtue of the fact that biosynthesis is needed to permit the appearance of the sympathetic transmitter. The accumulation of radioactively labeled norepinephrine is a measure of the availability of both axonal membrane transport sites and of storage sites for the catecholamine and these are to be found in sympathetic neurons (80). As a result of these findings, it was concluded that the density of sympathetic innervation and/or the uptake capacity of sympathetic nerves is not well developed in the newborn rat. The appearance and development of fluorescent adrenergic neurons within the rat heart seems to parallel the endogenous norepinephrine content described by others. An adult pattern for the distribution of cardiac adrenergic nerves appeared in the rat heart by the 22nd day after birth (137). However, the intensity of the green fluorescence (a measure of catecholamine concentration) and the density and thickness of fluorescent fibers continued to increase until about 35 days after birth. This corresponds reasonably well with the measurments of cardiac catecholamine content and ^{3}H-norepinephrine retention.

The development of sympathetic innervation in the rabbit heart is similar to that described for the rat. Norepinephrine and epinephrine contents were low in hearts isolated from fetal rabbits late in gestation on the 29th day (term is 31 days). At this time, norepinephrine averaged 0.11 ± 0.01 μg/g of heart while epinephrine averaged <0.10 μg/g of heart (54). The norepinephrine content increased continuously after birth to reach adult levels at about 3 weeks of age; epinephrine content remained unchanged. These results were confirmed and extended by Roffi and Motelica-Heino (132) who also found that cardiac norepinephrine content increased significantly within 4 hr after birth. Since norepinephrine content did not change between 4 and 72 hr after birth, it was proposed that the marked increase observed during the first 4 hr after birth may be related to stimulation of the cardiovascular and respiratory systems after delivery (132). The increments in cardiac norepinephrine content corresponded well with the development of fluorescent adrenergic nerves as demonstrated by the Falck-Hillarp histochemical method. Although fluorescent adrenergic fibers were detected in the fetal heart (29-day), the density was very low. Moderate innervation by adrenergic fibers characterized the hearts at 2 weeks after birth and the density of adrenergic innervation attained adult levels at 3 to 5 weeks after birth (54). The fetal and neonatal rabbit heart also contains small intensely fluorescent (SIF) cells that have been examined with the electron microscope (115). The small intensely fluorescent cells contained dense core vesicles (900 to 1800 Å diameter) in the cytoplasm and were usually found adjacent to blood vessels, particularly in the connective tissue around the aorta and pulmonary artery. These cells, which displayed synaptic densities with

neurons containing agranular vesicles (presynaptic cholinergic nerve?), have been considered secretory in nature and may serve as a source of catecholamines during a period of time (fetal and perinatal) when adrenergic neurons have not begun to function (115).

The human fetal heart is capable of synthesizing norepinephrine as early as the 13th week of gestation at a rate that does not change through the 23rd week (60). Measurements of tissue ability to synthesize norepinephrine may not indicate the presence of adrenergic neurons within the human fetal heart. For example, although fluorescent nerves can be demonstrated at greater density in atria than in ventricles of the human fetal heart at this time (unpublished observations cited by Gennser and V. Studnitz), the rate of norepinephrine synthesis was the same in atria and ventricles. Moreover, a study of catecholamine-containing cells in the human fetal heart with the Falck-Hillarp formaldehyde method revealed either no green fluorescent nerve trunks [8–18 weeks (39)] or a few intracardiac nerve trunks with a weak green fluorescence [10–16 weeks (124)]. Small intensely fluorescent cells were often detected in these preparations and these fluorescent cells were similar in appearance to those glomus-like cells found in sympathetic ganglia. The lack of terminal adrenergic nerve fibers coupled with the presence of the small intensely fluorescent cells prompted the conclusion that humoral adrenergic mechanisms rather than neural adrenergic processes modulated cardiac activity in the human fetus during the first half of pregnancy (39, 124).

The descriptions indicate that many mammals have a poorly developed adrenergic innervation of the heart at birth. However, this may not always be the case. In the guinea pig, sympathetic fibers entered the atrial wall from the 25th to the 29th days of gestation (72). A perimysial plexus of sympathetic fibers was present in ventricular muscle by 30 days; some of the fibers displayed the green fluorescence characteristic of adrenergic nerves treated according to the Falck-Hillarp method. It was concluded that autonomic innervation of the guinea pig heart is complete by the 30th day of gestation. However, fluorescent fibers were not abundant in the perimysial plexus until the 40th day of gestation and it is not known when these fibers are able to release transmitter. A comparative study of adrenergic neuron development with the Falck-Hillarp histochemical technique revealed a more rapid development in neonatal guinea pigs than in rats (98). The sheep also displays a rapid development of adrenergic fibers in the heart before birth (90). Large fluorescent nerve trunks grow into the heart along the coronary arteries and pass into the myocardium between 75 and 85 and 100 and 110 days gestational age. At term (150 days), well developed terminal sympathetic fibers are found around cardiac muscle cells. By contrast, most of the norepinephrine was reported in preterminal nerve trunks in another histochemical fluorescence study of the fetal sheep heart with the Falck-Hillarp technique (53). Cardiac norepinephrine content increased significantly between several weeks before term ($\sim$ 0.4 μg of norepinephrine/g of heart) to 3 days after birth ($\sim$ 1.2 μg/g); norepinephrine content did not differ between 3-day-old and adult sheep. The increase of norepinephrine content that occurred between several weeks before term and 3 days after birth was not accompanied by a change in the activity of tyrosine hydroxylase. However, activity of this enzyme increased significantly between 3 days after birth and adulthood, when norepinephrine content was unchanged. The significance of these reciprocal changes in activity of the rate-limiting enzyme and the content of the metabolic product in the sheep heart is unknown. On the basis of these biochemical measurements, it was concluded that sympathetic innervation of the sheep heart increased significantly in the postnatal period. In this connection, it will be

remembered that cardiac adrenergic transmission has been demonstrated in the fetal sheep (see section IV B).

The catecholamine content of a developing tissue determined histochemically or biochemically in tissue extracts, may not be directly related to the ability of the sympathoadrenal system to function (110). In this regard the function of the adrenal medulla in the calf and the lamb is illustrative. The calf displays adult levels of catecholamines at birth whereas the lamb has less than adult levels. The newborn calf was able to release only small amounts of adrenal catecholamines whereas the lamb released large amounts in response to nerve stimulation (35). The reactivity of the adrenal medulla to nerve stimulation seemed to parallel the maturity of the neonatal mammal.

B. Adrenergic Neuroeffector Transmission

Stimulation of intracardiac nerves evoked propranolol-sensitive acceleration of the chick sinoatrial pacemaker for the first time on the 21st incubation day (121). The period of pacemaker acceleration followed a period of inhibition due to the simultaneous excitation of intracardiac cholinergic nerves. Atropine, which blocked the inhibition caused by cholinergic nerve stimulation, allowed the detection of neurally-dependent acceleration of the pacemaker on the 19th incubation day (117). There is a delay of about 1 week between the onset of inhibitory and acceleratory neuroeffector transmission in the chick heart. Ontogeny seems to repeat phylogeny since adrenergic transmission to the heart does not appear in vertebrates until the level of bony fish whereas cholinergic transmission occurs in cartilaginous fish (26).

The development of acceleratory adrenergic neuroeffector transmission in the chick sinoatrial pacemaker is preceded by the development of fluorescent adrenergic nerves in this tissue (117, 120). [A delay between the appearance of fluorescent adrenergic nerves and the onset of adrenergic neuroffector transmission has also been observed in the neonatal mouse vas deferens (55). Adrenergic fibers were visible on the day of birth and their density and intensity increased with age; adrenergic transmission occurred for the first time at 18 days after birth.] Since the pacemaker reacts to catecholamines at this stage (see section IV C 1), the onset of adrenergic transmission was related in part, to the concentration of sympathetic transmitter at the postjunctional receptor. This position is supported by the results obtained in experiments done with the development of the positive chronotropic effect of an indirectly acting sympathomimetic agent, tyramine (109, 118). A detailed description of the actions of tyramine is given later (section IV C 2). The concentration of transmitter at the receptor depends upon the development of transmitter synthesis and of stimulus secretion coupling, the availability of transmitter in releasable stores, the diffusion of the released transmitter to the receptors, and the metabolism of released transmitter.

Field stimulation evoked a biphasic (negative followed by positive) inotropic effect in chick ventricles (18) and atria (10, 19) as early as 2 weeks after hatching. Although it is not known when adrenergic neuroeffector transmission to chick ventricles develops, these studies are of considerable help in establishing the pharmacological properties of neuroeffector transmission. As mentioned previously (section III B), the negative inotropic effect of field stimulation, which is antagonized by atropine, hyoscine, and triethylcholine, is due to stimulation of postganglionic cholinergic fibers to cardiac muscle. The positive inotropic effect of field stimulation is due to stimulation of intracardiac branches of postganglionic adrenergic nerves (10, 18, 19). For example, ganglion blocking agents (pempidine, mecamylamine, and hexamethonium) had no effect on the augmentation of the force of contraction

caused by field stimulation (18). The positive inotropic effect of field stimulation was antagonized by *beta*-receptor blocking agents (dichloroisoproterenol, pronethalol, propranolol) and by adrenergic neuron blocking agents (guanethidine, bretylium, choline-2,6-xylyl ether). Identification of the cardiac receptor as that of the *beta*-adrenergic type was strengthened by the fact that *alpha*-adrenergic receptor blocking agents did not diminish the positive inotropic effect of field stimulation (18, 19). The picture that emerges indicates that the adrenergic nervous system in the avian heart, early in its development as a neurosecretory unit, has many of the features of the adrenergic nervous system in adult mammals. There are some differences that bear consideration. Firstly, the identity of the adrenergic transmitter has been disputed. Sturkie and Poorvin (147) found that the adult avian heart contained considerable amounts of epinephrine and norepinephrine, as noted previously (77). The cardiac content of epinephrine decreased much more rapidly than that of norepinephrine in unstimulated perfused hearts. It was concluded that norepinephrine was the transmitter since it was the only catecholamine released during stimulation of cardiac sympathetic nerves and that epinephrine was located in an extraneuronal compartment (perhaps chromaffin cells). In experiments reported in 1975, De Santis *et al.* (44) excluded the possibility that all of the cardiac epinephrine was simply due to the presence of adrenal venous blood in the heart. Furthermore, sympathetic stimulation (electrical impulses, tyramine, elevated $(K^+)_o$) released norepinephrine and epinephrine into the perfusate in proportion (about 5:1) to their myocardial content. Treatment with 6-hydroxydopamine, which destroys adrenergic nerves, reduced the cardiac concentrations of norepinephrine and epinephrine; however, no experiments were done to determine the effect of this treatment on catecholamine release. It was concluded that epinephrine and norepinephrine are sympathetic neurotransmitters in the avian heart (44). No explanation has been given for the different results obtained in the two laboratories. The result obtained by De Santis *et al.* (44) are of phylogenetic interest because it places the bird between amphibia (epinephrine) and mammals (norepinephrine) with respect to cardiac sympathetic transmitter.

Secondly, cocaine did not intensify the inotropic effects of exogenously applied norepinephrine or of adrenergic nerve stimulation in the chick ventricle (18). However, Bennett and Malmfors (10, 11) reported that cocaine augmented the intensity and duration of the positive inotropic effect of adrenergic nerve stimulation in the left atrium of chicks 2 weeks after hatching. The discrepancy between these findings was attributed to the less dense innervation of the ventricle as compared to the atrium (9, 11). However, experiments done by Bolton and Bowman (19) in chick atria 2 to 4 weeks after hatching showed that cocaine did not intensify the positive inotropic effect of epinephrine. In these experiments, cocaine reduced the intensity of responses to tyramine. It is possible that the interpretation based on adrenergic neuron density is correct (see 11). This interpretation depends upon a large neuromuscular distance rendering a cocaine-sensitive neuronal uptake system inefficient. However, such an interpretation is not readily accepted in view of the observation that phenoxybenzamine, which can interfere with neuronal uptake in other structures, augmented the response to adrenergic nerve stimulation in the chick ventricle (18) as well as in the atrium (11).

Adrenergic transmission in the chick heart displays some other pharmacological properties that are of interest. Reserpine, in high doses, did not impair adrenergic transmission in the ventricle of 6-week-old chicks (18). Treatment with reserpine augmented the basal force of contraction presumably by a direct action on ventricular

muscle cells; this effect of reserpine was not antagonized by adrenergic neuron or adrenergic receptor blocking agents. The limited effectiveness of reserpine as an agent that interferes with adrenergic transmission does not seem to persist into adult life. Treatment of adult chickens with small doses of reserpine (0.2 mg/kg/day for 3 days) reduced the positive chronotropic response to sympathetic nerve stimulation to 40% of that observed in untreated animals (154). It is possible that higher doses are required in young chicks because the drug is more readily metabolized. An example of an adrenergic neuron blocking agent whose duration of action depends upon age is found in experiments done with 6-hydroxydopamine. Treatment of young (2 week) chicks with 6-hydroxydopamine was associated with the nearly complete disappearance of fluorescent terminal adrenergic nerves in the heart within 24 hr. Terminal adrenergic fibers first reappeared around 10 days after treatment; complete recovery of adrenergic nerves in atrial tissue was complete at 28 to 30 days after treatment (12). By contrast, complete reappearance of normal cardiac adrenergic nerve density in the pacemaker region required 40 to 50 days when 6-week-old chicks were used. The difference between the two groups of animals was attributed to a more efficient uptake system for 6-hydroxydopamine in adrenergic nerve terminal membranes of older chicks (12). This phenomenon was also observed in left atrial preparations from 2- and 6-week-old chicks. Recovery of the positive inotropic response of the left atrium to adrenergic nerve stimulation from the inhibitory effect of 6-hydroxydopamine occurred in parallel with the reappearance of fluorescent adrenergic nerves and both features of adrenergic nerve activity recovered more rapidly in 2-week-old chicks (10). Measurements of 6-hydroxydopamine uptake as a function of age are needed to support this proposal. In addition, one would also have to determine whether adrenergic nerves, injured by something other than 6-hydroxydopamine, regenerate at the same rate in 2- and 6-week-old chicks before accepting this hypothesis.

The onset of adrenergic transmission has not been studied systematically in the developing mammalian heart (135). Although the effects of autonomic blocking agents *in vivo* may indicate the occurrence of neuroeffector transmission, caution regarding the interpretation of such experiments has been given (135). Whereas there are few data regarding the onset of adrenergic neuroeffector transmission in the mammal, the experimental results illustrate the development of function in cardiac adrenergic neurons. Experiments done in mammals, like those done in birds, indicate that development of adrenergic neurons continues for some time after the neurosecretory ability of the cell and its role in neuroeffector transmission have been demonstrated.

Sympathetic transmission to the heart has been studied in the neonatal dog (57, 58). Stimulation of postganglionic sympathetic fibers of the right stellate ganglion in dogs within 1 day after birth accelerated the sinoatrial pacemaker (57, 58). The sympathetic innervation to the right atrium seemed to attain adult characteristics more rapidly than the sympathetic innervation to the ventricles. Moreover, a dichotomy between norepinephrine content and functional sympathetic innervation (transmission) was observed. Whereas ^{3}H-norepinephrine uptake per gram of tissue in the right atrium reached adult values at 10 days after birth, the norepinephrine content did not attain adult values until between 42 and 56 days after birth (57). Interestingly, sympathetic nerve stimulation increased heart rate to the same extent at 10 days as at 56 days and in adult animals. That is to say, sympathetic nerve function in the sinoatrial pacemaker seemed to develop in relation to the ability of the right atrium to take up ^{3}H-norepinephrine rather than with regard to norepinephrine content. In the dog

ventricle, ^{3}H-norepinephrine uptake and norepinephrine content developed in parallel and reached adult values at 56 days after birth. If neuroeffector transmission is related to the ability to take up ^{3}H-norepinephrine, sympathetic stimulation would not be expected to evoke ventricular responses characteristic of the adult until 56 days after birth. This view assumes that ^{3}H-norepinephrine uptake by a tissue is a more accurate index of adrenergic innervation than the norepinephrine content. However, it should be noted that the effects of nerve stimulation in the right atrium of the dog on day 10 may equal those on day 56, even though adrenergic neuroeffector transmission is quantitatively different. The response in young animals may reflect an immaturity of the sympathetic system due to the release of small quanta of transmitter, supersensitivity of the heart, and little reuptake of released transmitter by adrenergic nerves (58). In adult dogs, adrenergic transmission would be associated with the release of large quanta of transmitter, subsensitivity of the heart, and optimal reuptake of released transmitter. Whether disparities in the development of these three components can account for the following observation made by Boatman and Brody (17) is unsettled. Stimulation of the left sympathetic cardiac nerve accelerated the sinoatrial pacemaker in 1- and 2-week-old dogs as well as in adults but did not change heart rate in 4- and 8-week-old dogs (17). Perhaps stimulation of the right sympathetic cardiac nerve, as done in the studies of Gauthier *et al.* (57) and Geis *et al.* (58) would remove the discrepancy. The mammalian sinoatrial node is innervated predominantly by the right cardiac sympathetic nerve and this pattern may be established early in development. Results obtained in the neonatal lamb heart are illustrative in this connection (46). Stimulation of the left cardiac sympathetic nerve evoked a positive inotropic effect in the ventricle in all experiments but this procedure evoked a positive chronotropic effect in only 59% (10 out of 17) of the animals. It was concluded that the distribution of nerve fibers is typical, that is, that the sinoatrial pacemaker is influenced primarily by the right cardiac sympathetic nerve in the neonatal lamb.

Although functioning efferent adrenergic neuronal pathways to the heart were demonstrated in the neonatal dog, circulating catecholamines from the adrenal medulla seemed to have a greater role in modifying cardiac performance than did cardiac adrenergic nerves (58). A different pattern was observed in the neonatal lamb where sympathetic nerve stimulation evoked marked inotropic effects in ventricular muscle of the isolated, blood perfused heart (46). Stellate ganglion stimulation accelerated the fetal lamb heart at 70 days gestation (40) whereas splanchnic nerve stimulation did not release catecholamines from the adrenal medulla until 120 days gestation (35). These observations are consistent with the possibility that sympathetic modulation of cardiac activity in the lamb depends upon cardiac adrenergic nerves from early in fetal life. Comparison of the results of sympathetic nerve stimulation in the hearts of neonatal dogs and sheep supports the conclusion that functional adrenergic innervation of the heart develops more rapidly in animals that are relatively independent at birth.

Experiments have been done to determine the onset of adrenergic neuroeffector transmission in human fetal atria. Positive inotropic responses to field stimulation were evoked in hearts from fetuses 10 to 11.9 cm in length (10-cm length is equivalent to 13–14 weeks after fertilization). The positive inotropic effect, which followed a negative inotropic effect, reached a maximum within 15 sec after the end of stimulation and had dissipated within the next 30 to 90 sec (158). That excitatory transmission in the human fetal heart was adrenergic was also supported by pharmacological evidence that showed blockade of the positive inotropy by propranolol and augmentation of the positive inotropy by

cocaine and phenoxybenzamine. The ontogenetic appearance of adrenergic transmission, as in the chick heart, followed that of cholinergic transmission. The temporal relationship to cholinergic inhibitory transmission was also similar to that observed in the chick heart. However, the inconsistent occurrence of excitatory transmission may have been due to the effects of anesthesia and hypoxia (158) which render the results of such studies less subject to control (see below).

Cardiac adrenergic transmission in the rabbit heart was carefully studied in newborn (less than 24 hr after birth) and young rabbits (139). Acceleration of the sinoatrial pacemaker by sympathetic nerve stimulation was clearly related to activation of postganglionic adrenergic nerves since it was blocked by administration of guanethidine or bretylium. Acceleratory transmission was more sensitive to depression by anesthetic agent and by stimulus frequency in hearts from young (<1 week old) animals (139). Therefore, the use of anesthetic agents could have interfered with the ability to detect the onset of autonomic neuroeffector transmission as noted previously with respect to studies done on human fetal atria. Interestingly, the depression of adrenergic transmission to the pacemaker by elevated stimulus frequencies was attributed to conduction block caused by intracellular accumulation of Na^+ in the small diameter fibers. These findings suggest that metabolic factors, along with transmitter storage, release, and diffusion, can be rate-limiting in impulse transmission from nerve to cardiac muscle cell.

C. *Reactivity to Adrenergic Agonists and Antagonists*

1. Catecholamines. The chick embryo heart reacts to catecholamines very early in development. Markowitz (105) suggested that the positive chronotropic effect of epinephrine developed some time after the 2nd incubation day when an intermediary substance (receptor) appeared that allowed the reaction. Pacemaker acceleration by epinephrine was independent of the presence of nerves; epinephrine (10^{-8} to 10^{-7}g/ml) accelerated the chick embryo heart as early as the 37th incubation hours (74). These results were confirmed by Barry (5) who reported that the acceleration caused by epinephrine waned in the continued presence of the drug. He also noted, as did Fingl *et al.* (52), that the magnitude of the acceleratory effect of epinephrine was inversely related to the initial pacemaker rate; at control frequencies greater than 180 impulses per min, no positive chronotropic effect was observed. Additional support for the early appearance of the adrenergic receptor in pacemaker cells came from the experiments in which a positive chronotropic effect was induced by norepinephrine (109) and by isoproterenol (82) on the 2nd incubation day. Experiments done in Shideman's laboratory were the first to identify the pharmacological properties of the receptor in the chick embryo heart as those of the *beta*-adrenergic type (106). This conclusion has been confirmed in several laboratories (18, 82, 101). Thus, it is agreed that the chick embryo heart displays adrenergic receptors that mediate the positive chronotropic effect of catecholamines very early in development, that is, soon after spontaneous contractions have been observed (for a different view, *cf.* 112). Since spontaneous electrical activity of the pacemaker appears several hours before contractions (155), it is possible that the acceleratory effect of catecholamines is present before contractions develop. This has not been examined.

There is disagreement about the onset of the effect of catecholamines on contractility in the chick embryo heart (see 96). Experiments done by Shigenobu and Sperelakis (140) and by Hollman and Green (73) indicated that catecholamines did not evoke a positive inotropic effect in ventricular muscle from 4- to 7-day chick embryos. This conclusion is disputed by Shideman (106) who concluded that chro-

notropic and inotropic effects of catecholamines can be demonstrated on the 4th incubation day. Since heart rate was not controlled in Shideman's experiments, changes in rate caused by the catecholamines can complicate the interpretation of changes in contractility. Therefore, it seemed reasonable to conclude from experiments in which contraction rate was controlled that catecholamine-induced increases in ventricular contractility are not evident in 4- to 7-day chicks (73, 140). The sensitivity of electrically-driven 4-day ventricular preparations to elevation of external Ca^{++} (7.2mM) is very low (6, 73). It is possible that the low sensitivity to Ca^{++} and/or the low myofilament density is responsible for the limited reactivity of the ventricle to the positive inotropic effect of the catecholamines (3). This argument does not deal with the onset of the chronotropic effects of the catecholamines which is clearly evident at earlier stages.

The reactivity to catecholamines may change during development and there have been some attempts to study this issue in both avian and mammalian preparations. There are many variations in the experimental conditions that make it difficult to determine whether a change in reactivity has occurred.

An early study of the 2- to 5-day chick embryo heart concluded that variations in responsiveness of the pacemaker to epinephrine could not be related to age or to initial heart rate (74). Although Barry (5) suggested that the initial rate was an important factor in evaluating the occurrence of a positive chronotropic effect, he concluded that no marked change in pacemaker sensitivity occurred up to the 7th incubation day. This view was also held by Fingl *et al.* (52) and by McCarty *et al.* (106). These investigators concluded that morphological innervation, which was thought to occur at around the 5th incubation day, had no immediate effect on reactivity to catecholamines. In animals deprived of the hindbrain at 40 to 45 hr, and in which no efferent autonomic fibers were detected at the 9th incubation day, a positive chronotropic response to epinephrine was present (83). As mentioned previously (see section IVA), sympathetic neurons may not reach the heart until 9 to 10 days after fertilization and intra-axonal localization of transmitter can be detected at around the 12th incubation day.

Pacemaker sensitivity to isoproterenol and to epinephrine has also been studied in the chick heart at the time of onset of adrenergic neuroeffector transmission (101). Pacemaker reactivity to epinephrine and isoproterenol as indicated by the drug concentration that evoked half the maximum response (ED50) was the same on the 18th incubation day and the 21st incubation day (hatching). It is on the latter day that adrenergic neuroeffector transmission to the pacemaker can first be detected in untreated preparations. The pacemaker displayed subsensitivity to both catecholamines on the 19th and 20th incubation days. The cause of the subsensitivity is unknown. It seemed unlikely to be the result of an increased uptake of catecholamines by adrenergic nerves since isoproterenol was similarly affected (*cf.* 70). The subsensitivity may be due to intense activation of adrenergic receptors (see 42) by adrenal medullary hormones released during the hypoxia associated with the terminal stages of life *in ovo*. Girard (64) noted that the correction of the hypoxic conditions *in ovo* reversed the subsensitivity of the chick embryo to the vasopressor effects of catecholamines. Alternatively, increased metabolism by catechol-O-methyltransferase, whose levels increased at this time (76), may have caused the subsensitivity. The possibility that neuronal uptake of catecholamines may begin after hatching was suggested by the observation that the ED50 of isoproterenol decreased by 1 week after hatching whereas that of epinephrine did not change. It is of interest to note that the ED50 for the positive chronotropic effect of epinephrine was 0.26 μM and 0.39 μM on the day of hatching and 1 week later, respectively (101). These

values are close to those obtained for the positive inotropic effect of epinephrine in atria from 2-week (0.41 μM) and 6-week (0.61 μM) chicks (10).

Analysis of the role of neuronal uptake in the ability of the chick heart to take up catecholamines has given divergent results. The 5- to 9-day chick embryo heart accumulated ^{3}H-norepinephrine against a concentration gradient; the catecholamine appeared to be passively distributed in 3- and 4-day preparations (79). In addition, accumulation of ^{3}H-norepinephrine was impaired by cocaine, metabolic inhibitors, low temperatures and reserpine in 5- to 9-day, but not in 3- and 4-day, embryo hearts. The accumulation of ^{3}H-norepinephrine was attributed to uptake by sympathetic nerves that appeared on the 5th incubation day. As mentioned previously, innervation of the heart by postganglionic sympathic fibers may not occur until the 9th or 10th incubation days and fluorescent nerve fibers in the atrial plexus are not evident until the 12th incubation day (see section IVA). Resolution of this matter clearly requires experiments designed to identify the presence of nonfluorescent intracardiac sympathetic nerves and the relationship between catecholamine accumulation and identified nerves. It would be helpful to determine how much of the ^{3}H-norepinephrine taken up by the heart is retained as a function of time after "loading." Experiments such as this were done in the neonatal rat heart (65) and it was shown that the accumulated ^{3}H-norepinephrine was more readily washed out of the young (1–21 day) heart.

The reactivity to catecholamines during development has been studied in several mammals. Cardiac reactivity to norepinephrine and epinephrine was found to change during development of the fetal rat (13). Norepinephrine (10^{-5} g/ml) accelerated the sinoatrial pacemaker on the 13th but not the 16th or 19th to 21st gestational days. Epinephrine (10^{-5} g/ml) accelerated the pacemaker on the 19th to 21st but not on the 13th or 16th gestational days. The mechanism involved in the transition of reactivity to these catecholamines has not been studied. Although the effects of the catecholamines were attributed to activation of *beta*-adrenergic receptors, no tests with adrenergic blocking drugs were done. Pharmacological tests involving adrenergic receptor blocking agents would be especially helpful in determing the mechanism involved in the transition of reactivity to these catecholamines. It is noteworthy that an earlier study of adrenergic reactivity reported that the fetal rat heart responded to epinephrine as early as day 10½ of gestation (69). Hall (69) conducted his experiments at 37.5°C whereas Bernard and Gargouïl (13) did their experiments at 24°C. The divergent results of these two studies conceivably could be reconciled in experiments designed to examine the effect of temperature on reactivity to exogenously applied catecholamines. This has been suggested by Robkin *et al.* (131) who confirmed the experimental findings of Hall (69) regarding the appearance of adrenergic reactivity on day 10 in preparations maintained at 37°C. Results obtained in the amphibian heart clearly illustrate the importance of temperature in determining the pharmalogical characteristics of adrenergic reactivity (88). The adrenergic receptor in the fetal rat heart is activated by isoproterenol and blocked by propranolol (131). These results, along with those obtained by Hall (69), suggest that catecholamines interact with a *beta*-adrenergic receptor in the fetal rat heart. It is noteworthy that acceleration by isoproterenol appeared at least one-half day earlier than did the inhibition by carbamycholine. Whether the onset of carbamylcholine-induced inhibition is not readily detected in preparations with a low pacemaker frequency remains to be determined. It would be of interest to determine whether carbamylcholine has an inhibitory effect in preparation accelerated by isoproterenol.

Norepinephrine and isoproterenol accelerated the sinoatrial pacemaker of the

mouse heart as early as the 13th to 14th gestational day (160). However, it is not known whether the sensitivity to catecholamines changes with age since the maximum acceleration evoked by the drugs increased with age. Furthermore, the concentrations required to evoke half-maximum effects were not reported. [Parenthetically, it is of interest that sensitivity to drugs of organs other than the heart may not change during ontogenesis as shown in the work of Boréus and McMurphy (20). The pD_2 value for the acetylcholine-induced contractions of guinea pig ileum did not change from the last week of gestation through adult life; however, the maximum tension increment evoked by the drug increased as muscle efficiency increased (20).] It is helpful to consider the results obtained in the rabbit heart in connection with those described for the mouse. The reactivity of the rabbit heart to norepinephrine, as indicated by the ED50 for the positive chronotropic effect, did not change from birth to 3 months (25). There are no data regarding the time of onset of adrenergically-mediated pacemaker acceleration during development and whether the reactivity to catecholamines changes up to the time of birth. In addition, it would be helpful to use isoproterenol as the test substance since the likelihood of its being taken up by the neuronal transport system is limited (70).

There are some examples among mammals of sensensitivity to catecholamines that develops with increasing age. Reactivity of the sinoatrial pacemaker of the newborn dog heart to isoproterenol and to norepinephrine was nearly the same (isoproterenol was slightly more potent). The concentration-effect relationships for both amines shifted to the right in adult dogs (58). The subsensitivity of the adult heart can be viewed as the result of at least two processes operating at pre- and postsynaptic sites, respectively, the subsensitivity due to the appearance of a neuronal transport system that can accumulate norepinephrine from the extracellular fluid and the subsensitivity of the receptor mechanism due to continual bombardment by transmitter (*cf.* 42). In order to test these suggestions, experiments should be done with isolated hearts from neonatal and adult dogs in the presence and absence of cocaine. Alternatively, the reactivity of the chronically denervated (sympathectomy) adult heart could be compared with that of the neonatal animal to determine whether the aforementioned mechanisms are adequate explanations for the "supersensitivity" of the neonatal heart to catecholamines.

There are no differences in cardiac inotropic effects of norepinephrine and isoproterenol in lambs from birth to 5 days of age (see 46). However, as the authors mention, the problem had not been studied systematically. In 1972, Friedman (53) concluded that the sensitivity of the *beta*-receptor mechanism activated by isoproterenol was the same in fetal (last 15 days of gestation) and adult sheep ventricular muscle. However, the relationship between developed tension and norepinephrine concentration shifted to the right over this time period. Accordingly, the apparent subsensitivity of the adult heart to norepinephrine was attributed to neuronal uptake of norepinephrine (53). However, subsensitivity of the receptor mechanism itself was not readily demonstrable in the sheep heart. The operation of this mechanism cannot be evaluated until evidence is available concerning the onset of adrenergic neuroeffector transmission in sheep ventricular muscle. Adrenergic transmission begins before birth of the lamb since postganglionic sympathetic nerve stimulation accelerated the sinoatrial pacemaker in the fetal lamb at 70 days gestation (40).

Reactivity to catecholamines has been demonstrated in the human fetal heart as early as the 9th week of gestation (59) Epinephrine was reported to be a more potent stimulant of cardiac contractions than norepinephrine in perfused human fetal hearts at 16 to 24 weeks gestational age (4). In 1972, it was noted that the

human fetal heart (12–22 weeks) was more sensitive to isoproterenol than to norepinephrine (31). These findings suggest the presence of *beta*-adrenergic receptors in the human fetal heart. Although Coltart and Spilker (31) concluded that the reponse of human fetal atria to isoproterenol increased with age from 12 to 22 weeks, it is not possible to draw any conclusions about the sensitivity of the *beta*-adrenergic receptor during this time. Information regarding sensitivity requires experiments in which the normalized concentration-effect relationship is studied in tissues that display different tension or rate maxima.

2. *Tyramine.* The pharmacology of cardioactive drugs that are capable of interacting with autonomic nerves is of particular interest in a study of the development of autonomic cardiac innervation. Tyramine has been used to evaluate the participation of endogenous catecholamines in the reactivity of the heart to drugs. Conflicting results have been obtained in experiments done in the chick embryo heart. Lelorier *et al.* (95) found that 10^{-6} M tyramine increased the rate and force of spontaneous contractions in chick hearts isolated on the 7th incubation day but not on the 4th incubation day. These results were attributed to the presence, in the older embryos, of the sympathetic nervous sytem which released norepinephrine in response to the addition of tyramine (95). In a more systematic study, Michal *et al.* (109) found that tyramine (11.5×10^{-6} M) had no stimulating effect on the chick embryo heart until the 16th incubation day. In contrast to the results obtained by Lelorier *et al.* (95), the drug had no effect on the heart from the 2nd through the 8th incubation day. The augmentation of the rate and displacement of spontaneously contracting preparations caused by tyramine increased continually up to 3 days after hatching and these effects of tyramine were diminished in animals treated with reserpine. Results obtained in our laboratory indicate that tyramine has a positive chronotropic effect in isolated chick embryo atria as early as the 11th incubation day (118). The pacemaker acceleration was less than 20 impulses per min until the 20th incubation day when tyramine increased average pacemaker frequency by more than 60 impulses per min. When the decline in initial rate observed late in ontogenesis (102) was considered in the evaluation of the positive chronotropic effect of tyramine, the results from our laboratory agree with those obtained by Michal *et al.* (109) with respect to percentage increases.

The marked increase in the pacemaker acceleratory effect of tyramine, which was blocked by cocaine or by propranolol, was attributed to the appearance of transmitter stores in adrenergic neurons that developed in the sinoatrial pacemaker region (117, 118). The slight acceleration evoked by tyramine from the 11th through the 19th days could be due to a direct action of the drug on pacemaker cells or to the release of catecholamines from non-neuronal stores. This conclusion conflicts with that given by Lelorier *et al.* (95) but agrees with that given by Michal *et al.* (109). In this regard, the effects of nicotinic drugs can also be considered. Nicotine and dimethylphenylpiperazinium accelerated the sinoatrial pacemaker after the onset of adrenergic transmission (21st and 28th days) but not beforehand [15th incubation day (118)]. The positive chronotropic effect of nicotine drugs required elevation of Ca^{++} to 3.6 mM (twice normal). These results were attributed to an improvement, by Ca^{++}, of the coupling between nicotine stimulus and secretion of transmitter by cardiac adrenergic nerves in the chick heart. The stimulatory effect of nicotinic drugs on avian sympathetic nerves seems to diminish between the time of hatching and adult stages and it has been proposed that nicotinic excitatory receptors are not an integral component of the terminal portion of the sympathetic neuron (49). In the adult chicken, nicotinic drugs (nicotine, acetylcholine), in the presence of

atropine, neither stimulated the heart nor released catecholamines into the coronary perfusate. Dimethylphenylpiperazinium had sympathomimetic effects that were attributed to a tyramine-like action because the release of norepinephrine by the drug was not impaired by reduction of external Ca^{++} and the drug did not evoke antidromic discharges over cardiac sympathetic nerves (49). Since nicotinic drugs had sympathomimetic effects in atria (118) and ventricles (18) of atropine-treated chicks (day of hatching and 3 weeks later), the lack of sympathomimetic effect of nicotinic drugs in the adult chicken heart may be due to a paucity of nicotinic excitatory receptors (49) and/or to a labile coupling between nicotinic stimulus and transmitter secretion.

Tyramine augmented the force of contraction in atrial (10) and in ventricular (18) muscle strips isolated from hatched chicks. The positive inotropic effect was reduced by treatment with cocaine (19), 6-hydroxydopamine (10), and reserpine (19). These observations support the conclusion that the sympathomimetic effect of tyramine in the chick heart depends upon release of adrenergic transmitter. The fact that the positive inotropic effect of tyramine and the fluorescence of terminal adrenergic neurons are reduced by 6-hydroxydopamine and reappear concomitant with each other and with the restoration of adrenergic transmission is strong support for the neural origin of the adrenergic transmitter released by tyramine (10, 12).

Tyramine increased the rate and force of cardiac contractions in newborn (1–5 day) lambs; the increments in these parameters were the same in 2-month-old (46). The adrenal medulla was eliminated experimentally as a source of catecholamines during the administration of tyramine and the sympathomimetic effect of the drug was attributed to release of norepinephrine from cardiac adrenergic nerves. Tyramine accelerated the heart of the adult dog twice as much as that of the neonate (58). The positive chronotropic effect of tyramine (60 μg/kg, intravenously) was equal to that caused by 0.07 μg of norpeinephrine per kg in the neonatal dog and about 1 μg of norepinephrine per kg in the adult dog. It was speculated that tyramine evoked greater acceleration of the adult heart by releasing more transmitter from cardiac adrenergic nerves (58). (Alternatively, the greater effect of tyramine in adult dogs could be due to release of more catecholamines from extracardiac sources.) A contrasting hypothesis has been given to explain the effect of tyramine on the rabbit heart. Tyramine increased sinoatrial pacemaker frequency in the perfused hearts of newborn rabbits (25). The ED50 for the positive chronotropic effect of tyramine was shifted to the right in adult rabbits. Brus and Jacobowitz (25) attributed this finding to release of more norepinephrine from adrenergic nerves in the newborn rabbit heart as compared to the adult rabbit heart. Measurements of the amount of norepinephrine released by tyramine would help determine the validity of the hypotheses for the changes in the actions of tyramine (25, 58). It is also possible that the adrenergic receptor may be subsensitive in the hearts of older rabbits. It should be noted that the sympathomimetic effect of tyramine on the hearts of newborn sheep and rabbits was attributed to release of norepinephrine from intracardiac adrenergic nerves. There is little or no pharmacoligical evidence to support this assumption. It would be of interest to determine when the sympathomimetic effect of tyramine appeared during development of these mammals. It would be expected that the onset of adrenergic transmission evoked by electrical and chemical stimulation of the nerves would coincide. Results obtained in fetal mouse hearts exposed to tyramine are of interest in this connection. Wildenthal (160) noted that tyramine, in concentrations ranging from 10^{-6} M to 10^{-3} M, had no effect on sinoatrial pacemaker frequency in hearts isolated from animals on the 13th to 20th gestational days. Tyramine (10^{-4} M) evoked a significant acceleration of the pacemaker for the first time on

the 21st to 22nd gestational days. Because the fetal mouse heart displayed a positive chronotropic effect to isoproterenol and to norepinephrine at least 1 week before tyramine accelerated the pacemaker, it was tentatively concluded that the effect of tyramine was indirect. If the marked increase in the action of tyramine were the result of an indirect effect mediated by release of neuronally located adrenergic transmitter, it is possible that the onset of the chronotropic effect of tyramine signals the appearance of adrenergic neuroeffector transmission. This possibility could be tested with application of electrical stimuli to excite intracardiac nerves.

D. Mechanism of Transmitter Action

The ionic mechanism for the pacemaker acceleration caused by catecholamines in the embryonic heart is not known. Atrial pacemaker activity in the adult vertebrate heart has been attributed to a time-dependent decrease in an outward current carried largely, but not exclusively, by K^+ (24). Epinephrine increased the intensity of an inwardly directed Ca^{++} current and of the outwardly directed current in the atrial pacemaker. Pacemaker acceleration caused by epinephrine was due to an increase in the magnitude of the inward current; the increased outward current would limit the amount of acceleration. Although it is difficult to conduct voltage clamp experiments on embryonic heart muscle, it should be possible to determine whether the pacemaking mechanism found in adult atrial cells also operates in the embryo heart with the aid of constant current techniques and ionic substitution experiments.

The positive inotropic effect of catecholamines in the chick embryo heart has been attributed to an increase in membrane conductance to Ca^{++} (140). The catecholamines restored action potentials to ventricular muscle cells whose early transient conductance (Na^+) was blocked by tetrodotoxin or inactivated by elevated K^+. These action potentials, which are termed "slow action potentials," are releated to the generation of the plateau phase of the cardiac action potential and to the initiation of contraction (reviewed in 130 and 153).

Cyclic 3′,5′-adenosine monophosphate (cyclic AMP), its dibutyryl derivative and methylxanthines restored "slow action potentials" to chick embryo ventricular cells whose early transient (Na) conductance had been blocked by tetrodotoxin (133). That is, the effects of these cyclic nucleotides and phosphodiesterase inhibitors were similar to those of exogenously applied catecholamines. These results are of interest because of the relationship of the "slow action potentials" to an inward Ca^{++} current and the development of tension (see above). Polson *et al.* (129) reported that norepinephrine and isoproterenol increased cyclic AMP and the force of contraction in hearts isolated from chicks on the 4th and 7th incubation days (129). These results have not been obtained by others. Hollman and Green (73) showed that addition of catecholamines, which increased cyclic AMP levels in 7- to 9-day but not in 4-day embryonic hearts, evoked a positive inotropic effect only in the 7- to 9-day hearts (73). This finding has been confirmed recently by McLean *et al.* (107) who also confirmed the observation (73) that basal levels of cyclic AMP decreased during ontogenesis. A causal relationship between the inotropic effect of the catecholamines and cyclic AMP has not been established. For example, isoproterenol increased the force of contraction in ventricular muscle from hatched chicks (1–7 days) but it did not increase cyclic AMP (73). McLean *et al.* (107) reported isoproterenol-induced increments in cyclic AMP were lower in 18- to 19-day than in 7- to 8-day embryo hearts. Whereas isoproterenol increased the force of contraction in the 7- to 8-day embryo hearts, no data are given for this parameter in 18- to 19-day hearts. McLean *et al.* (107) also reported that the cyclic AMP levels of heart cells cultured from 16-day hearts were about twice as high as those in intact preparations from this day. This increase in cyclic AMP has been related to the phenomenon

in which heart cells in culture acquire the electrophysiological properties of younger embryonic heart cells, including tetrodotoxin resistant "slow action potentials." However, this proposal is not readily supported in view of the quantitative disparities in cyclic AMP levels reported. For example, heart cells from the 11th to 12th days have higher cyclic AMP levels than those cultured from the 16th day and yet the former retain tetrodotoxin-sensitive action potentials. This objection might be answered if information were available concerning the cellular disposition of cyclic AMP rather than content.

Epinephrine and norepinephrine stimulated adenylate cyclase in particulate preparations obtained from the hearts of fetal sheep (2), mice, and rats (160, 161). Although an increased force of contraction by catecholamines was measured in fetal rodent hearts (161), it has not been determined whether there is a causal relationship between catecholamine-induced activation of adenylate cyclase and of contraction. It is of interest that 10^{-3} M dibutyryl cyclic AMP, which can enter cells, had only a slight deceleratory effect on the sinoatrial pacemaker in 19- to 22-day fetal mouse hearts (160). Studies of the relationship between the effect of catecholamines on force and cyclic AMP in human fetal hearts have yielded divergent results. Norepinephrine, epinephrine, and isoproterenol activated adenylate cyclase in human fetal hearts as early as 6 to 7 weeks of gestation when the whole homogenate (114) rather than a high speed particulate fraction was used as source of the enzyme (39). The procedures used to make the high speed particulate fraction for measurement of adenylate cyclase activity in human embryonic hearts may have inactivated or destroyed the adrenergic receptor (114). Adenylate cyclase activity of the partiuclate fraction was increased by fluoride. The results obtained in the study of the human fetal heart by Dail and Palmer (39) disagree with those obtained by Coltart *et al.* (33). The latter group (33) reported that adenyl cyclase activity increased from the 12th to 22nd gestational week whereas the former (39) found that enzyme activity declined. These conflicting results suggest that use of human hearts for experimental purposes may be particularly dissatisfying, not only because of the moral and ethical questions raised but also because of the problems associated with recovery from the effects of pre-anesthetic drugs, anesthetic agents, and the isolation of the fetus.

Summary

The development of neuroeffector transmission in the embryo and fetal heart requires the appearance and function of presynaptic and postsynaptic components. During ontogenesis, function of the postsynaptic component (transmitter receptor and membrane conductance mechanism) can be demonstrated before the presynaptic component (efferent autonomic nerve) can be detected in the heart. The properties of the postsynaptic receptor have been identified with classical pharmacological methods. Receptor-specific ligands have been used to determine the chemical properties of muscarinic (75) and *beta*-adrenergic (67) receptors in tissues from adult animals. It is hoped that receptor-specific ligands, particularly those derived from antagonists, can be employed successfully to measure the chemical properties of these receptors in the developing heart. In addition, more information is required to enable us to understand the role that ontogenetic changes in membrane ion conductance and intracellular cyclic nucleotides have in determining the actions of neurotransmitter on pacemaker and contracting cardiac cells.

The results of morphological studies of the development of the cardiac autonomic nervous system do not always agree with the results obtained in biochemical and histochemical analyses of nerve function. A lack of quantitative information and the observation of divergent results from different laboratories are the difficulties en-

countered in achieving the synthesis of structural and functional properties of developing nerves. Although neuroeffector transmission has been a useful index of the onset and development of neurosecretory function, chemical determination of the amount of transmitter released by stimuli is required to determine the development of neurosecretory activity and its relationship to the ontogenetic appearance of neuroeffector transmission. Direct measurement of transmitter release would be essential in order to study the development of neuronal interactions, for example, muscarinic inhibition of adrenergic transmitter release (see 100).

There is no conclusive evidence to support the possibility that changes in transmitter sensitivity are due to the onset of neuroeffector transmission. Experiments in which developmemt of the autonomic nervous system is accelerated or decelerated by various procedures (changing egg incubation temperature, administration of ganglion blocking drugs, nerve growth factor and its antiserum) are essential to determine whether the changes in transmitter sensitivity that occur are causally related to the onset of neurosecretory (or neuronal transport) function.

The morphological and functional development of postganglionic adrenergic nerves seems to be regulated by intracellular and extracellular events (see 61, 151). However, it is not known to what extent presynaptic and effector cell development are able to regulate the growth and differentiation of the postganglionic neuron. It has been proposed that trans-synaptic factors regulate the development of the adrenergic nerve terminal and of the effector cells innervated by the adrenergic nerves (16). Furthermore, it has been speculated that a trophic influence of the effector cell on neuronal input can regulate the biochemical development of postganglionic cholinergic nerves (29) and the retrograde transport of nerve growth factor in adrenergic nerves (123). The exploration of these problems in the development of cholinergic and adrenergic nerves of the heart is a stimulating challenge.

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