0022-3565/84/2302-0419$00.00/0
THE JOURNAL OF PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS

Vol. 230, No. 2
Printed in U.S.A.

Ontogeny of Cardiac Ornithine Decarboxylase and its *Beta* Adrenergic Responsiveness in the Rat

SUSAN P. MISKA,[1] GARY L. KIMMEL, JAMES R. HARMON and PEGGY WEBB

Developmental Mechanisms Branch, Division of Teratogenesis Research, National Center for Toxicological Research, Jefferson, Arkansas

Accepted for publication May 14, 1984

ABSTRACT

Ornithine decarboxylase (ODC) is a marker of tissue growth and development and, because sympathetic stimulation of *beta* adrenergic receptors acutely increases ODC in the adult rat heart, measurement of this enzyme can be used to indicate the functional intactness of the *beta* adrenergic receptor system in the heart. Changes in the postnatal ontogenetic pattern of this enzymatic activity may also indicate abnormal development and ODC appears to be particularly useful in evaluating the effects of prenatal insult on cardiac development. The present study examines the pattern of basal ODC activity and its developing sensitivity to *beta* adrenergic stimulation during the perinatal period in order to establish a data base for studies on the effect of various environmental agents on the developing cardiovascular system. ODC activity was measured in rat hearts on gestation day (GD) 20 through postnatal day (PND) 28 under saturating conditons of L-ornithine and pyridoxal 5-phosphate. Basal ODC activity fell from 3 nmol of CO_2/hr/mg of protein at GD 20 to less than 0.5 nmol of CO_2/hr/mg of protein at PND 18, rising again to nearly 1 nmol of CO_2/hr/mg of protein at PND 22. The *beta* adrenergic agonist isoproterenol (10 mg/kg s.c.) resulted in peak ODC stimulation at 4 hr postinjection on PNDs 6, 14 and 21; however, no response was seen at PND 1 at this dose or at GD 20 (300 μg/kg s.c.). In dose-response studies, isoproterenol produced a maximal response at 10 mg/kg s.c., resulting in increases from control of 67, 230 and 1700% at PNDs 6, 14 and 21, respectively, indicating that the sensitivity of the heart to *beta* adrenergic stimulation increases with age, during the perinatal period.

Development of the rat heart occurs over a considerable portion of the pre- and early postnatal period and appears to be influenced significantly by the process of cardiac sympathetic innervation. Sympathetic innervation and central nervous control are not complete until after birth in the rat (Pappano, 1977; Bareis and Slotkin, 1978; Seidler and Slotkin, 1979; Slotkin, 1979). However, heart rate is responsive to direct *beta* adrenergic stimulation early in development (Robkin *et al.*, 1976) and development of sympathetic connections appears to be occurring throughout the pre- and early-postnatal period (Pappano, 1977). Thus, the normal development of the heart and its sympathetic control may be sensitive to prenatal insult by environmental agents. Many substances known to affect cardiovascular function do so by altering the sympathetic nervous system control of the heart and embryonic treatment with such drugs has been shown to result in cardiovascular abnormalities. For example, in the chick embryo the appearance of cardiovascular anomalies has been correlated with the increasing *beta* adrenergic activity of the sympathomimetic amine to which it was exposed (Hodach *et al.*, 1974, 1975; Gilbert *et al.*, 1977). In rodents, caffeine and salicylates, both of which can alter adrenergic function, have been shown to cause cardiovascular defects if given prenatally (Takacs and Warkany, 1968; Fujii and Nishimura, 1974).

Our laboratory is interested in the effects of prenatal exposure on the developing heart and its neural input. ODC appears to be a rate-limiting enzyme in the synthesis of polyamines and its activity is associated with growth and differentiation (Russell and Snyder, 1968; Russell, 1980). In addition, sympathetic stimulation of *beta* adrenergic receptors increases acutely ODC activity in the adult heart and measurement of ODC can be used to indicate the functional intactness of the *beta* adrenergic system in the heart (Slotkin, 1979). Thus, the ontogenetic pattern of ODC becomes a specific marker of the development of a tissue and a shift in this pattern after environmental insult can indicate a disturbance of normal developmental processes (Anderson and Schanberg, 1972, 1975; Slotkin *et al.*, 1976; Slotkin, 1979). The current study examines further the pattern of basal ODC activity during the perinatal period and the developing sensitivity of this enzyme to direct *beta* adrenergic stimulation.

Received for publication November 28, 1983.
[1] Present address: Department of Physiology and Pharmacology, Philadelphia College of Osteopathic Medicine, Philadelphia, PA 19131.

ABBREVIATIONS: ODC, ornithine decarboxylase; GD, gestation day; PND, postnatal day; TED buffer, 50 mM Tris-HCl-2 mM disodium EDTA-5 mM dithiothreitol; BSA, bovine serum albumin.

Materials and Methods

Methods. Sprague-Dawley-derived female CD rats (approximately 100 days old) were random-bred at the National Center for Toxicological Research (Jefferson, AR) animal colony. The animals were housed in animal rooms maintained at 22–24°C and 40 to 60% relative humidity on a 12-hr light cycle. They received laboratory chow (Purina no. 5012) and water *ad libitum*. The morning that copulation plugs were found was designated GD 0. Pups from all litters born before 4:30 P.M. on either GD 21 or GD 22 were randomized at birth and redistributed to the nursing mothers. This day was designated as PND 0. Litter size was kept at 10 pups of the same sex.

In fetal studies, pregnant rats (GD 20) were injected s.c. with saline (0.9%) or isoproterenol or left untreated. At the appropriate time, the dams were sacrificed by CO_2 and cervical dislocation, followed by removal of the fetuses. The fetal hearts were removed, rinsed in ice-cold saline, blotted, dissected free of connective tissue and hand-homogenized in 20 volumes of ice-cold TED buffer (pH 7.2 at 25°C). Neonatal studies differed only in that animals were killed by cervical dislocation alone and the hearts were minced before homogenizing in 10 volumes of TED buffer. The injection schedule was designed to minimize time-of-day and treatment effects and required less than 1.5 hr to complete. In both approaches, the homogenate was transferred to a 5 × 20 mm polyallomer tube and centrifuged (Beckman Airfuge, prechilled A-100/18 rotor) at 148,000 × *g* for 10 min at 25°C. The resultant supernatant fraction (cytosol) was assayed for protein with the Bio-Rad Protein Assay (BSA used as a standard) and for ODC activity as described below. Assay blanks in tubes containing TED buffer instead of cytosol were subtracted to correct for nonenzymatic CO_2 release.

ODC activity was measured by the release of $^{14}CO_2$ from L-[1-^{14}C] ornithine (Russell and Snyder, 1968). The assays were performed in 15-ml Corex centrifuge tubes capped with a Kontes rubber stopper with suspended polypropylene center well. The incubation mixture consisted of 50 μl of cytosol, 25 μl of reaction mixture [final concentrations of 0.05 mM pyridoxal 5-phosphate and 2 mM L-(+)-ornithine monohydrochloride prepared in TE buffer (Tris-HCl, 50 mM; disodium EDTA, 2 mM; pH 7.2 at 25°C)] and 25 μl of 0.1 mM L-[1-^{14}C]ornithine to give a final volume of 100 μl. In preliminary experiments, these L-ornithine and pyridoxal 5-phosphate concentrations were found to give maximum ODC activity across the age-range tested. The tubes were incubated 1 hr at 37°C and the reaction was terminated by injecting 500 μl of 2 M citric acid through the rubber stopper into the incubation mixture. Agitation of the tubes was continued for an additional 30 min at 37°C to facilitate complete absorption of the evolved $^{14}CO_2$ into 200 μl of a 2:1 (volume) mixture of ethanolamine- 2-methoxyethanol contained in the center well. The center wells were placed into 10 ml of Scintisol scintillation cocktail and assayed for radioactivity in a Packard liquid scintillation spectrometer at a counting efficiency of 93% for ^{14}C.

In studies to determine the substrate saturation of ODC, postnatal rats of specific ages were injected with saline or isoproterenol (10 mg/kg s.c.) and were sacrificed 4 hr later. For each age, hearts from three male animals for each treatment group were pooled and centrifuged (Beckman ultracentrifuge, model L5-75) at 300,000 × *g* for 1 hr. The cytosol from each group was then assayed in the presence of varying L-ornithine concentrations (0.004–5.0 mM).

Materials. L-[1-^{14}C]Ornithine monohydrochloride (40–60 mCi/mmol) was purchased from New England Nuclear (Boston, MA); ethanolamine, 2-methoxyethanol (both reagents scintillation grade), pyridoxal 5-phosphate and L-(+)-ornithine monohydrochloride from Eastman Kodak Co. (Rochester, NY); Tris-HCl from Schwarz-Mann (Orangeburg, NY); disodium EDTA, citric acid and sodium chloride from Fisher Scientific Company (Springfield, NJ), BSA from Sigma Chemical Company (St. Louis, MO); dithiothreitol from Aldrich Chemical Company (Milwaukee, WI); Scintisol from Isolab, Inc. (Akron, OH); and Bio-Rad protein assay dye reagent concentrate from Bio-Rad Laboratories (Richmond, CA). Isoproterenol HCl used in animal injections was obtained through the National Toxicology Program (Research Triangle Park, NC).

Results

Basal ODC patterns. The pattern of basal cardiac ODC activity during the perinatal period is shown in figure 1. When assayed in the presence of saturating concentrations of L-ornithine and pyridoxal 5-phosphate, the basal activity (expressed per milligram of protein) was highest before birth and decreased approximately 80%, reaching low levels at PND 18. As expected, heart weight increased during this period of time; however, the rate of heart growth decreased and paralleled the decrease in ODC activity. Two specific developmental periods that were examined included the time around birth and the late preweaning period. To determine if parturition had an effect on basal ODC, activity was measured before (GD 22) and immediately after (0–6, 6–16, 24 and 48 hr) birth. As shown in figure 1, parturition did not significantly alter ODC levels. During the late preweaning period, there was a secondary rise in ODC activity at approximately PND 20 and this higher level of activity was maintained at least through the next week.

Because fluctuations in ODC activity within a specific 24-hr period could bias the time course studies on isoproterenol stimulation, ODC activity was measured at various times during the day on GD 20 and PNDs 6, 14 and 21. As shown in figure 2, the variability in activity is greatest at the earlier ages, with little or no fluctuation by PND 21. Whereas no consistent variation can be seen across ages, the pattern of activity at any one age seems to be consistent. At GD 20, there is a noticeable fluctuation in activity with levels peaking around 1200 hr and then declining over the next 2 hr. By PNDs 6 and 14, this fluctuation is decreased progressively and by PND 21 there is no fluctuation in ODC activity during the 6-hr time interval assessed in this study.

ODC-isoproterenol stimulation. ODC activity after isoproterenol stimulation was measured at five ages during the perinatal period. Figure 3 shows the pattern of the response

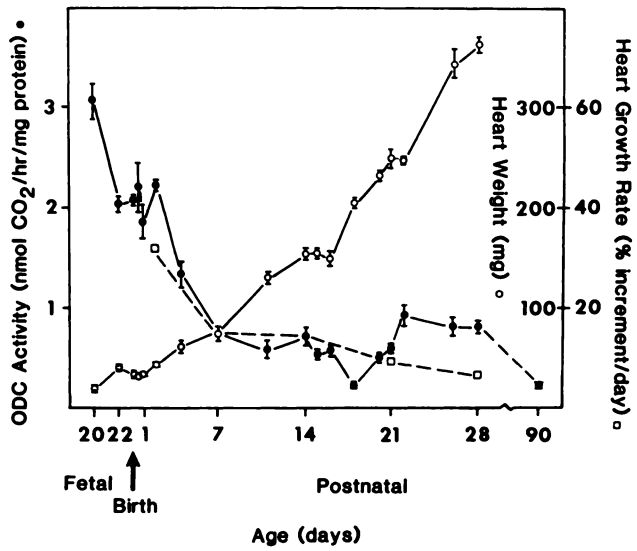

Fig. 1. Ontogeny of basal ODC activity and weight in fetal and postnatal rat hearts. Each ODC activity is the mean ± S.E.M. of multiple determinations on 2 to 10 hearts of mixed sexes. Heart weights are mean ± S.E.M. of all hearts used in the ODC determination. Heart weight increment is the percent change per day from the last point; PND 2 is change from GD 20.

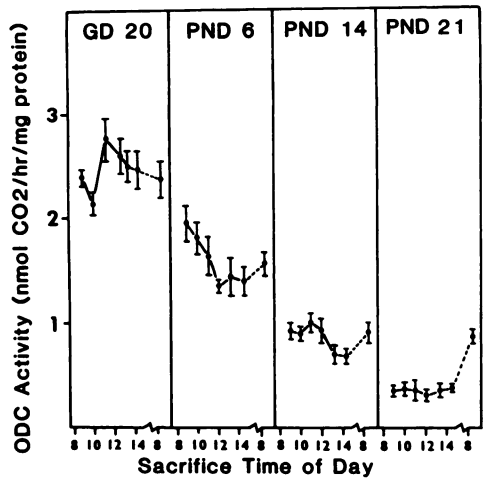

Fig. 2. Variation in basal ODC activity in hearts from rats on GD 20 and PNDs 6, 14 and 21. Each point is the mean ± S.E.M. of ODC activity in the hearts of 5 to 14 rats of mixed sexes. Results from untreated and saline-treated control groups were not different and therefore were combined for data analysis.

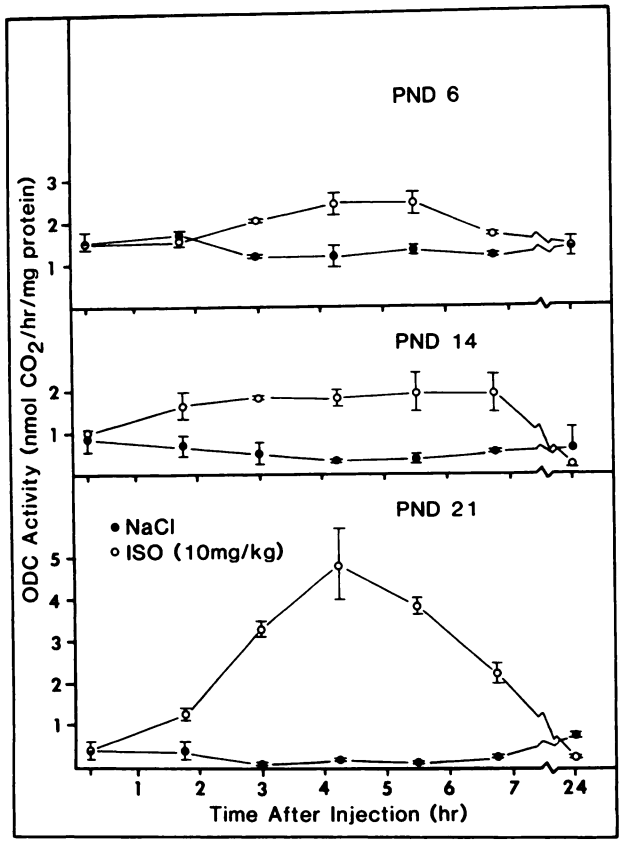

Fig. 3. Time course of cardiac ODC activity in control (●, saline-treated) or isoproterenol (ISO)-treated (○, 10 mg/kg s.c.) rats on PNDs 6, 14 and 21. Each point is the mean ± S.E.M. of ODC activity in the hearts of two to four rats injected between 7:30 and 9:00 A.M. and sacrificed at the indicated time after injection.

with time after s.c. administration of 10 mg/kg isoproterenol. On PND 6, the earliest test day on which isoproterenol stimulation of ODC could be demonstrated, the response was shallow, being first detectable at 3 hr after injection, plateauing between 4 and 6 hr and decreasing before 7 hr. The response on PND 14 began earlier and lasted longer than that on PND 6, with the plateau of activity maintained over at least 5 hr. By PND 21, a typical adult pattern was noted, with activity peaking at approximately 4 hr after stimulation and no extensive plateauing. Responsiveness of ODC activity to isoproterenol stimulation also was evaluated on PND 1 (10 mg/kg s.c.) and GD 20 (300 μg/kg s.c.) and there was no obvious stimulation at any of the time points measured (data not shown). Studies at higher isoproterenol doses in prenatal animals were not possible because of significant maternal toxicity.

The dose-response effect of isoproterenol (0.1–100 mg/kg) on ODC activity was measured 4 hr after injection in hearts at PNDs 6, 14 and 21 (fig. 4). On PND 6, an elevation of activity (67%) above that in the unstimulated heart was seen only at the 10-mg/kg dose. By PND 14, an increase in activity could be detected at 1.0 mg/kg and, although the absolute level of activity at 10 mg/kg was not as high as that on PND 6, the increase in activity (230%) was considerably higher, due to the substantial decrease in basal ODC activity between PNDs 6 and 14. By PND 21, the dose-response nature of the stimulation was distinct. There was a slight (not significant) increase detectable at 0.1 mg/kg, a significant increase at 1 mg/kg and a 1700% increase in activity under maximal stimulation (10 mg/kg). At all ages, the 100-mg/kg dose resulted in the same or a smaller stimulation than at 10 mg/kg of isoproterenol. Although the reasons for this are unknown, Haddox *et al.* (1981) have described a similar decrease in ODC stimulation in the late-gestation fetal mouse heart and have suggested that it is possibly related to a drug-induced cytotoxicity.

Having demonstrated that isoproterenol stimulation of L-ornithine saturated ODC activity increases with age, additional studies were carried out to determine whether age and isoproterenol pretreatment could change ODC affinity for L-ornithine. The effects of isoproterenol stimulation on L-ornithine saturation kinetics were compared on PNDs 6 and 21. On PND 6, the unstimulated enzymatic activity displayed saturation kinetics with a K_m of 0.095 mM and a V_{max} of 1.55 nmol of CO_2/hr/mg of protein. After isoproterenol stimulation, there was a small increase in V_{max} to 2.34 nmol of CO_2/hr/mg of protein, but no change in K_m (0.093 mM). By PND 21, the K_m increased to 0.143 mM and the V_{max} decreased to 0.36 nmol of CO_2/hr/mg of protein. After isoproterenol stimulation on PND 21, there was a shift in the K_m to 0.073 mM and a marked increase in the V_{max} to 4.58 nmol of CO_2/hr/mg of protein.

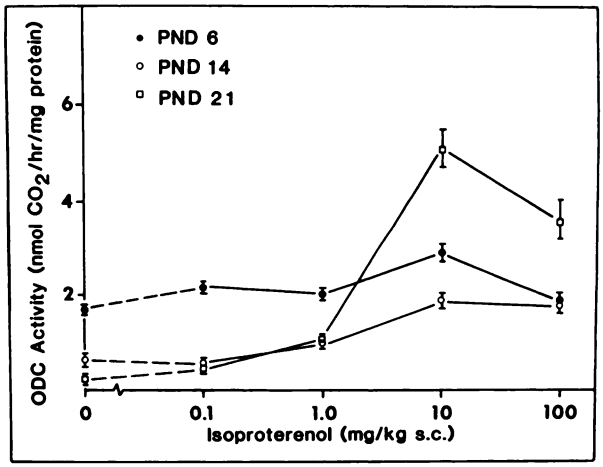

Fig. 4. Effects of increasing isoproterenol dose on cardiac ODC activity in hearts removed from rats on PNDs 6, 14 and 21, 4 hr after treatment. Each point is the mean ± S.E.M. of ODC activity in 7 to 12 hearts from rats treated as indicated.

Discussion

The present study has examined the pattern of basal ODC activity during the perinatal period and the developmental sensitivity of this enzyme to direct *beta* adrenergic stimulation. ODC is considered a marker for general growth and development and the pattern of a declining basal cardiac ODC activity from the late fetal to the early postnatal period (fig. 1) (Anderson and Schanberg, 1972) is indicative of a significant change in cardiac development. This is supported by the parallel decrease in the cardiac growth rate (fig. 1) and is consistent with the findings of Claycomb (1975), who demonstrated that cardiac DNA synthesis decreases dramatically after birth and virtually ceases by the middle of the 3rd postnatal week. Moreover, the demonstration of a secondary rise in ODC activity that occurs in the heart at PND 18 (fig. 1) coincides with the finding of Claycomb (1977) that cardiac growth shifts from hyperplasia to hypertrophy between 2 and 3 weeks of postnatal age. Claycomb (1977) has suggested that the shift may be related to the inhibition of DNA synthesis in response to the establishment of functional adrenergic innervation that occurs during this same early postnatal period (see Pappano, 1977). If this is the case, then the secondary rise in ODC activity may be in response to an increased control through adrenergic neural inputs.

The pattern of the daily fluctuation in cardiac ODC activity is dependent on age, being more variable during the late pre- and early postnatal period and decreasing progressively over the next 3 weeks (fig. 2). At birth, there was no significant spike in activity, indicating that the event of birth does not alter basal ODC in the heart and confirming the findings of Anderson and Schanberg (1972). This is in contrast to their findings in the brain in which birth produces a sharp spike in basal ODC activity. The results of the current study emphasize the importance of study design, especially at the younger neonatal ages. Careful consideration of injection and sacrifice times and animal randomization may be necessary to avoid any bias associated with daily fluctuations.

As it relates to our general interest in the neural control of the developing heart, we were particularly interested in establishing the pattern and timing of *beta* adrenergic stimulation of cardiac ODC. Stimulation of ODC activity by the *beta* adrenergic agonist, isoproterenol, has been shown in this study to be an age-related event, both in its appearance and its magnitude (figs. 3 and 4). There was a marked increase in activity on PND 21, with a peak of activity at 4 hr after stimulation and a return to base-line values by at least 24 hr. These results are similar to those reported for the adult rat, where maximal stimulation ranges from 6- to 11-fold greater than base-line values and occurs between 2 and 4 hr postinjection (Warnica *et al.*, 1975; Lau and Slotkin, 1982). This adult pattern, however, is not apparent at the earliest ages tested in this study, as stimulation was not achieved at any of the doses used at either GD 20 or PND 1. This suggests that the connection between the *beta* adrenergic receptor and the ODC response is not functional at this developmental stage, although metabolic or pharmacokinetic events which reduce the effective concentration of agonist reaching the receptor may be involved. These results also indicate that there is a separation of the trophic and chronotropic response of the heart to *beta* adrenergic receptor stimulation, as heart rate is increased by *beta* adrenergic agonist stimulation as early as GD 10 (Robkin *et al.*, 1976).

By PND 6, the sensitivity of ODC to isoproterenol stimulation is evident, but only at the maximal stimulating dose (10 mg/kg). In contrast, Bartolome *et al.* (1977) have reported stimulation on PND 4 using 0.1 mg/kg of isoproterenol. The reason for the discrepancy in these two studies relative to the dose required for stimulation is not apparent. However, both studies demonstrate that a functional *beta* adrenergic receptor-ODC connection is established within the 1st week of postnatal life and our results indicate further that this connection requires a considerable portion of the preweaning period to become fully functional.

The alterations in ODC affinity for L-ornithine reported here are consistent with those obtained by Lau and Slotkin (1979), in that there is a decrease in affinity with increasing age and an increase in affinity after isoproterenol stimulation at PND 21. These shifts may represent developmental or stimulation-related changes in the enzyme. Lau and Slotkin (1979) have noted high- and low-affinity forms in PND 2 hearts and adult hearts after isoproterenol stimulation. Thus, both from results of the present study and those of Lau and Slotkin (1979), the affinity of the enzyme appears to be correlated positively with periods of cardiac growth and increased ODC activity, although the specific mechanism by which these alterations in ODC affinity occur is as yet undefined. It should be noted, however, that although the affinity of ODC for L-ornithine appears to be changing with age, this factor cannot be responsible for the increase in ODC response to isoproterenol seen with age in the present study. ODC was assayed under saturating conditions of L-ornithine and therefore is a measure of total enzyme present and is independent of the affinity of the enzyme for L-ornithine. These affinity changes, however, may be important physiologically when L-ornithine availability as a substrate is limited.

The decrease in V_{max} of ODC activity between PND 6 and PND 21 in saline-treated rats found when examining the effects of age and isoproterenol on L-ornithine kinetics is the same as that found in those studies designed specifically to measure alterations in ODC activity with age. Similarly, the greater increase in V_{max} after isoproterenol at PND 21 than at PND 6 in these kinetic experiments can be seen in the isoproterenol time course and dose-response studies in these age animals. This is expected because these time courses and dose-response studies were all run in the presence of saturating L-ornithine and are measurements of the change in the V_{max} of the enzyme. If less than saturating L-ornithine had been used, then changes in measured ODC activity would not reflect accurately changes in V_{max} when comparisons were made between enzymes with different affinities such as from neonatal and adult hearts or from hearts from adult animals with and without isoproterenol pretreatment.

In conclusion, the developmental factors determining cardiac responsiveness to sympathetic input have been summarized as: 1) establishment of sympathetic innervation, 2) development of *beta* adrenergic receptors and 3) responsiveness of the heart to receptor stimulation (Bartolome *et al.*, 1977; Slotkin, 1979). The current study has focused on this latter aspect and has examined specifically the developmental pattern of ODC activity and its responsiveness to *beta* adrenergic stimulation. The results support the view that ODC activity is increased during periods of active hyperplasia. In addition, ODC activity also is

elevated during the 3rd postnatal week and this may be associated with the maturation of functional neural connections and the cellular hypertrophy that are occurring at this time. Furthermore, the results support the view that *beta* adrenergic receptor-ODC coupling occurs sometime during the 1st week of postnatal life, as well as demonstrate that the magnitude of stimulation that occurs at this time is not maximal and matures over the next 2 weeks. With this data base, studies are being initiated on the effect of prenatal exposure to various agents on the developing heart and its responsiveness to direct *beta* adrenergic stimulation; preliminary results on prenatal exposure to propranolol and reserpine have been reported (Buelke-Sam *et al.*, 1983; Harmon *et al.*, 1983).

Acknowledgments

The authors wish to express their appreciation to Ms. E. Sykes for her assistance in the preparation of this manuscript.

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Send reprint requests to: Dr. G. L. Kimmel, HFT-134, Developmental Mechanisms Branch, Division of Teratogenesis Research, National Center for Toxicological Research, Jefferson, AR 72079.