1976

The functional development of the adrenal-sympathetic nervous system in neonatal and adolescent swine

Sidney Koon Hung Woo

University of the Pacific

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THE FUNCTIONAL DEVELOPMENT OF THE ADRENAL-
SYMPATHETIC NERVOUS SYSTEM IN NEONATAL
AND ADOLESCENT SWINE

A Thesis
Presented to
the Faculty of the Graduate School
University of the Pacific

In Partial Fulfillment
of the Requirements for the Degree
Master of Science

by
Sidney K. Woo
December 1976
DEDICATION

The author dedicates this thesis to his mother, who is an infinite source of inspiration to her children.
ACKNOWLEDGMENTS

The author wishes to express his sincere gratitude to Dr. Hubert C. Stanton for his invaluable guidance and support throughout his encouragement in this research; Dr. Donald Y. Shirachi, for his encouragement, guidance, and advice; Dr. Marvin H. Malone for his supervision and advice in the preparation of this thesis; Dr. Charles W. Roscoe and Dr. Horach H. Loh for generously agreeing to act as members of his thesis committee.

The author is also grateful to Mr. Raymond L. Mueller and Dr. Harold J. Mersmann of Shell Development Company for their interest and help in this research.

Finally, the author wishes to express his appreciation to Mrs. Carol Sarnoff for her technical assistance in the preparation of this thesis.
TABLE OF CONTENTS

LIST OF TABLES ........................................ vii
LIST OF FIGURES ...................................... ix

Chapter

I INTRODUCTION ...................................... 1

Statement of the Problem ............................ 1
Review of the Literature .............................. 5

Catecholamines and the Adrenal Medulla ............. 5
Ontogenes is of the Sympathetic Nervous System ....... 22
Perinatal Survival of the Swine ..................... 25

II MATERIALS AND METHODS .......................... 30

Animals ................................................. 30
Estimation of Medullary and Cortical Volumes ........ 30
Determination of Catecholamines ..................... 32
Determination of Catecholamine Biosynthetic Enzymes Activities ........................................ 34

Tyrosine Hydroxylase (TH) ......................... 36
DOPA Decarboxylase (DDC):
  Method 1 ............................................. 39
DOPA Decarboxylase (DDC):
  Method 2 ............................................. 39
Dopamine-ß-hydroxylase (DBH) ...................... 42
Phenethanolamine-ß-Methyltransferase (PNMT) ......... 45
Biuret Method ......................................... 48
Estimation of Maximal Velocity (Vmax) and Michaelis Constant (Km) ......................................... 49

Determination of Tyrosine Concentrations in Adrenal Gland and Plasma .......................... 49
<table>
<thead>
<tr>
<th>Chapter</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>II</td>
<td></td>
</tr>
<tr>
<td>Tyrosine Extraction from Adrenal Glands.</td>
<td>49</td>
</tr>
<tr>
<td>Tyrosine Extraction from Plasma.</td>
<td>50</td>
</tr>
<tr>
<td>Fluorometric Determination of Tyrosine Concentration.</td>
<td>50</td>
</tr>
<tr>
<td>In Vitro Biosynthesis of Catecholamines from Tyrosine by Adrenal Slices</td>
<td>51</td>
</tr>
<tr>
<td>Amine Uptake by Adrenal Vesicles</td>
<td>52</td>
</tr>
<tr>
<td>Amine Efflux from Adrenal Vesicles</td>
<td>58</td>
</tr>
<tr>
<td>III</td>
<td>59</td>
</tr>
<tr>
<td>RESULTS</td>
<td></td>
</tr>
<tr>
<td>Growth of the Adrenal Gland: General Aspects</td>
<td>59</td>
</tr>
<tr>
<td>Relationship Between Adrenal and Body Weight</td>
<td>59</td>
</tr>
<tr>
<td>Growth of the Medulla in Relationship to the Cortex</td>
<td>59</td>
</tr>
<tr>
<td>Relationship Between Catecholamine Content and Piglet Age</td>
<td>62</td>
</tr>
<tr>
<td>Ontogenic Patterns of Catecholamine Biosynthetic Enzymes</td>
<td>69</td>
</tr>
<tr>
<td>Tyrosine Hydroxylase (TH) Activity and Piglet Age</td>
<td>69</td>
</tr>
<tr>
<td>DOPA Decarboxylase (DDC) Activity and Piglet Age</td>
<td>69</td>
</tr>
<tr>
<td>Dopamine-ß-Hydroxylase (DBH) Activity and Piglet Age</td>
<td>73</td>
</tr>
<tr>
<td>Phenethanolamine-ß-Methyltransferase (PNMT) Activity and Piglet Age</td>
<td>73</td>
</tr>
<tr>
<td>Apparent Kinetic Patterns of the Catecholamine Biosynthetic Enzymes and Piglet Age</td>
<td>73</td>
</tr>
<tr>
<td>Synthesis of Catecholamines by Adrenal Slices in vitro</td>
<td>78</td>
</tr>
<tr>
<td>Effect of α-Methyl-ß-Tyrosine (αMpt) on the in vitro Biosynthesis of Catecholamines</td>
<td>78</td>
</tr>
<tr>
<td>Adrenal and Plasma Tyrosine Concentrations and Piglet Age</td>
<td>80</td>
</tr>
<tr>
<td>Ontogenesis of in vitro Catecholamine Biosynthesis</td>
<td>80</td>
</tr>
<tr>
<td>Chapter</td>
<td>Summary</td>
</tr>
<tr>
<td>---------</td>
<td>---------</td>
</tr>
<tr>
<td>III</td>
<td>Ontogenesis of <em>in vitro</em> Amine Uptake and Efflux by <em>Isolated</em> Adrenal Vesicles. 83</td>
</tr>
<tr>
<td></td>
<td>Relationship of Amine Uptake with Piglet Age. 83</td>
</tr>
<tr>
<td></td>
<td>Relationship of Amine Efflux with Age. 88</td>
</tr>
<tr>
<td>IV</td>
<td>DISCUSSION. 92</td>
</tr>
<tr>
<td>V</td>
<td>SUMMARY. 106</td>
</tr>
<tr>
<td></td>
<td>BIBLIOGRAPHY. 108</td>
</tr>
</tbody>
</table>
LIST OF TABLES

Table | Page
---|---
I Piglet Mortality at Five Locations | 3
II Diet Compositions, Percent | 31
III Percent of Medullary Volume in Terms of Cortical Volume as Related to Species and Age | 61
IV Mean Body Weights and Adrenal Gland Catecholamine (CA) Content Correlated with Piglet Age | 63
V Mean Specific Activity of Tyrosine Hydroxylase in Adrenal Homogenates Correlated with Piglet Age | 70
VI Mean Specific Activity of DOPA Decarboxylase in Adrenal Homogenates Correlated with Piglet Age | 70
VII Mean Specific Activity of Dopamine-β-Hydroxylase in Adrenal Homogenate Correlated with Piglet Age | 74
VIII Mean Specific Activity of Phenelthanolamine-N-Methyltransferase in Adrenal Homogenate Correlated with Piglet Age | 74
IX Apparent Michaelis-Menten Kinetic Parameters of Catecholamine Biosynthetic Enzymes in Adrenal Homogenate Correlated with Piglet Age | 77
X Mean Tyrosine Concentration in Plasma and Adrenal Glands Correlated with Age | 82
XI Mean Catecholamine Synthesis Correlated with Tyrosine Concentration and Piglet Age | 84
LIST OF TABLES  
(continued)

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>XII</td>
<td>Effect of Piglet Age on Vesicular Uptake of Epinephrine (10 min. incubation) in the Presence or Absence of Reserpine.</td>
<td>85</td>
</tr>
<tr>
<td>XIII</td>
<td>Effect of Piglet Age on Vesicular Uptake of Metaraminol (10 min. incubation) in the Presence or Absence of Reserpine.</td>
<td>86</td>
</tr>
<tr>
<td>XIV</td>
<td>Slopes of Uptake Curves Correlated with Age</td>
<td>89</td>
</tr>
<tr>
<td>XV</td>
<td>Efflux (60 minutes) of Epinephrine and Metaraminol Correlated with Piglet Age</td>
<td>91</td>
</tr>
<tr>
<td>Figure</td>
<td>Description</td>
<td>Page</td>
</tr>
<tr>
<td>--------</td>
<td>-----------------------------------------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>1</td>
<td>Norepinephrine standard curve.</td>
<td>35</td>
</tr>
<tr>
<td>2</td>
<td>Epinephrine standard curve</td>
<td>35</td>
</tr>
<tr>
<td>3</td>
<td>Determination of optional amount of homogenate, incubation time, and substrate concentration for the assay of tyrosine hydroxylase.</td>
<td>37</td>
</tr>
<tr>
<td>4</td>
<td>Determination of optional amount of homogenate, incubation time and substrate concentration for the assay of DOPA decarboxylase.</td>
<td>40</td>
</tr>
<tr>
<td>5</td>
<td>Determination of optional amount of homogenate, incubation time and substrate concentration for the assay of dopamine-β-hydroxylase.</td>
<td>43</td>
</tr>
<tr>
<td>6</td>
<td>Determination of optional amount of homogenate, incubation time and substrate concentration for the assay of phenethanolamine-N-methyltransferase.</td>
<td>46</td>
</tr>
<tr>
<td>7</td>
<td>Elution pattern of norepinephrine and normethanephrine.</td>
<td>53</td>
</tr>
<tr>
<td>8</td>
<td>Determination of optimal parameters of the amine uptake by isolated adrenal vesicles.</td>
<td>55</td>
</tr>
<tr>
<td>9</td>
<td>Relationship between body and adrenal weights.</td>
<td>60</td>
</tr>
<tr>
<td>10</td>
<td>The change of norepinephrine content as a function of age.</td>
<td>64</td>
</tr>
<tr>
<td>11</td>
<td>The change of epinephrine content as a function of age.</td>
<td>66</td>
</tr>
<tr>
<td>Figure</td>
<td>Description</td>
<td>Page</td>
</tr>
<tr>
<td>--------</td>
<td>-----------------------------------------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>12</td>
<td>The change of catecholamine content as a function of age.</td>
<td>67</td>
</tr>
<tr>
<td>13</td>
<td>The change of epinephrine content as a function of age.</td>
<td>68</td>
</tr>
<tr>
<td>14</td>
<td>The change of specific activity of tyrosine hydroxylase as a function of age</td>
<td>71</td>
</tr>
<tr>
<td>15</td>
<td>The change of specific activity of DOPA decarboxylase as a function of age</td>
<td>72</td>
</tr>
<tr>
<td>16</td>
<td>The change of specific activity of DOPA-β-hydroxylase as a function of age</td>
<td>75</td>
</tr>
<tr>
<td>17</td>
<td>The change of specific activity of phenethanolamine-N-methyltransferase as a function of age</td>
<td>76</td>
</tr>
<tr>
<td>18</td>
<td>In vitro inhibition of tyrosine hydroxylase by α-methyl-β-tyrosine.</td>
<td>79</td>
</tr>
<tr>
<td>19</td>
<td>In vitro inhibition of catecholamine biosynthesis by α-methyl-β-tyrosine.</td>
<td>81</td>
</tr>
<tr>
<td>20</td>
<td>Amine uptake by adrenal vesicles of 0-day-old swine with and without reserpine</td>
<td>87</td>
</tr>
</tbody>
</table>
Chapter I

INTRODUCTION

Statement of the Problem

Birth is a severe environmental change. The neonate is abruptly exposed to the harsh extrauterine environment and must seek food and shelter on its own. Furthermore, it must maintain homeostasis successfully in order to survive. The condition of homeostasis has been defined by Cannon (1939) as an internal state of equilibrium established by automatic adjustments when the animal experiences environmental changes. These internal adjustments are brought about by a number of coordinated physiological and biochemical mechanisms.

The development of the animal after birth is marked by complex changes. Certain periods are particularly important and have been defined as "critical periods" (Krecek, 1971). Two processes are important to the survival of the animal during these times. The process of "adaptation" involves the maturation of specific physiological systems according to the needs of the neonatal animal, and is dictated by the external environment. The process of
"tolerance" renders the neonate relatively unresponsive to a stressor as compared to the adult and protects the animal from over-responding and depleting vital resources until it is physiologically mature. An example of tolerance is the resistance to anoxia of immature neonates.

The newborn pig has a high rate of mortality. The number of pigs born 114 days after conception represent only about 55% of the original ova released. Besides having a large number of embryo losses during the gestation period, 13-25% of the live-born piglets die before weaning as shown in Table I.

The low viability of the neonatal piglets is undoubtedly related to their inability to maintain homeostasis during stress. The sympathetic division of the autonomic nervous system is a major coordinator of homeostasis. Therefore, a study of the development of the sympathetic nervous system could provide some clues to explain the high mortality rate of the newborn pigs. Since the coordinating ability of the sympathetic nervous system is not only dependent on the maturity of the system, but also on the development of the effector organs and their responsiveness to activation, it is logical to examine their relative development. Three possibilities can exist:

1. The sympathetic nervous system in neonatal pigs is underdeveloped relative to its effector organs. Homeostasis of the animal would then be poorly
TABLE I
Piglet Mortality at Five Locations

<table>
<thead>
<tr>
<th>Location</th>
<th>Farrowings, N</th>
<th>Litter Size, Mean</th>
<th>Stillborn, Percent</th>
<th>Death Prior to weaning, Percent</th>
<th>Total Loss, Percent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Minnesota</td>
<td>5,263</td>
<td>9.2</td>
<td>5.6</td>
<td>20.5</td>
<td>26.1</td>
</tr>
<tr>
<td>Indiana</td>
<td>1,302</td>
<td>9.7</td>
<td>5.5</td>
<td>13.0</td>
<td>18.5</td>
</tr>
<tr>
<td>United Kingdom</td>
<td>90</td>
<td>10.8</td>
<td>7.9</td>
<td>24.4</td>
<td>32.3</td>
</tr>
<tr>
<td>Canada</td>
<td>773</td>
<td>8.9</td>
<td>7.2</td>
<td>16.4</td>
<td>23.6</td>
</tr>
<tr>
<td>Illinois</td>
<td>21,536</td>
<td>9.8</td>
<td>7.0</td>
<td>19.7</td>
<td>26.7</td>
</tr>
</tbody>
</table>

aU.S.A. average loss = 20-30% of live-born pigs farrowed (Stanton and Carroll, 1974). The end result is 7.5 pigs/litter weaned.

coordinated when the piglet encounters environmental stressors. Consequently, the neonate would have difficulty in adaptation.

(2) The sympathetic nervous system in neonatal pigs develops at the same rate as its effector organs. Homeostasis would then be maintained in harmony and all the physiological systems would be fully utilized.

(3) The effector organs in neonatal pigs are underdeveloped relative to the sympathetic nervous system. The coordinating activity of the sympathetic nervous system would then be hampered because of the low responsiveness of these organs. Furthermore, the neonate would be unable to tolerate environmental changes because the sympathetic nervous system could exert excessive demands on the effector organs, thereby depleting the vital resources of the animal.

It is logical to postulate that the low capability of the neonatal piglets to cope with environmental stressors is due to a "dyssynchrony" of the development of the sympathetic nervous system and its effector organs. A number
of developmental studies on one type of effector organ--the metabolic depots--have been reported on the swine. The following studies were undertaken to determine the functional maturation of the sympathetic nervous system in order to allow this hypothesis to be tested. These studies were conducted using adrenal glands. The justification for this comes from the following observations:

1. When the sympathetic nervous system is activated by the cold, epinephrine has been observed to increase more markedly than norepinephrine during the period of initial challenge, suggesting a marked discharge from the adrenal medulla (Bertin and Chevillard, 1967).

2. The high percentage of epinephrine found in newborn swine exposed to cold stress implies significant contributions of catecholamines from the adrenal medulla (Stanton et al., 1972).

3. Severe stress activates the adrenal medulla in most animals (Yuwiler, 1971).

Review of the Literature

Catecholamines and the Adrenal Medulla

Structure of the Adrenal Medulla. The adrenal medulla is ontogenically, structurally and functionally similar to
the sympathetic ganglia. The nerves supplying the adrenal medulla terminate in deeply invaginated synapses in continuity with the chromaffin cells. Most of these fibers are believed to be cholinergic. Morphological evidence for this claim was initially based upon observations under light microscopy (Coupland and Holmes, 1958) and more recently demonstrated by electron histochemistry (Lewis and Shute, 1969). As these nerves are myelinated preganglionic fibers, the chromaffin cells are believed to be similar in anatomy and function to the postganglionic sympathetic neurons. In addition, it has been demonstrated that the norepinephrine-containing cell populations are innervated by postganglionic adrenergic fibers from an as yet unidentified ganglionic source (Prentice and Wood, 1975).

The adrenal medulla is composed of groups and columns of chromaffin cells interspersed with groups of isolated nerve cells and permeated with venous sinusoids. All the chromaffin cells are of the large columnar type arranged in rows one cell thick along the margins of the sinusoids. The bases of these cells point away from the sinusoids to the extracellular spaces where they synapse with the nerve terminals (Al-Lami, 1970). The adrenal gland is highly vascular, supplied by arteries derived from the abdominal aorta, the inferior phrenic and renal arteries (Coupland, 1965a). The venous sinusoids drain into the suprarenal vein, which emerges from the hilus of the adrenal gland.
and joins the inferior vena cava on the right and the left renal vein on the left.

**Histology of the Chromaffin Cell.** The fine structures of adrenal chromaffin cells have been described over the years for various mammalian species (Coupland, 1965a; Yates, 1963). A full spectrum of subcellular organelles is known to occur in these cells. These organelles are well developed, indicating the presence of high metabolic activity (Al-Lami, 1970). They include the mitochondria (slightly elongated), the Golgi complex and lysosomes. The chromaffin cells of young animals have numerous ribosomes arranged in groups which may have a helical or rosette form (Coupland and Weakley, 1968, 1970). Newborn animals have concentric arrays of rough endoplasmic reticulum whereas in older animals these are more thinly distributed or stacked in small groups (Coupland and Weakley, 1970). There is also evidence for the presence of secretory tubules and an intracellular cytoskeleton separating the catecholamine vesicles (Rahwan et al., 1973). The chromaffin cells have been classified into two types, namely the epinephrine- and the norepinephrine-storing cells (Coupland and Hopwood, 1966). The peripheral sympathetic nerve ending, in contrast, contains exclusively norepinephrine. Adult adrenal chromaffin cells store either epinephrine or norepinephrine granules under normal circumstances. However, granules from both of these catecholamines may be observed within the same
cell during fetal life and shortly after birth (Coupland and Weakley, 1970). The organelles of importance in the chromaffin cells are the membrane-bound, electron-dense vesicles which are the major storage sites for the catecholamines (Blaschko and Welch, 1953). The composition of these vesicles includes protein, lipids, catecholamines, adenosine phosphates, calcium, magnesium and water (Hillarp, 1959).

The soluble components of the vesicles contain adenine nucleotides with ATP being the major one. In addition, two of the soluble proteins have been identified. They are dopamine-β-hydroxylase and chromogranin A, which may be chemically related since purified bovine adrenal medullary chromogranin A has been reported to have the identical amino acid sequence to a subunit of soluble vesicular dopamine-β-hydroxylase (Aunis et al., 1975). The membrane of the chromaffin vesicle is made up of proteins which include dopamine-β-hydroxylase, ATPases, cytochrome B 561 and a cytochrome B:NADH reductase. It also contains almost all of the lipids in the vesicle (Kirshner, 1974).

**Catecholamine Biosynthesis.** Catecholamines are biogenic amines which possess a 3,4-dihydroxyphenyl (catechol) nucleus. The three most commonly found catecholamines in animals and plants are epinephrine, norepinephrine and dopamine. Recent investigations have indicated that a fourth, namely epinine, is found in bovine and porcine adrenal tissues (Laduron et al., 1974).
Epinephrine was the first catecholamine to be discovered (Abel and Crawford, 1897) and synthesized (Stolz, 1904). Norepinephrine was discovered and simultaneously synthesized by Stolz (1904). Following the discovery of DOPA decarboxylase (Holtz et al., 1938), the formation of norepinephrine was suggested to be part of the synthetic pathway of epinephrine (Blaschko, 1939). The hypothetical pathway of conversion of tyrosine into epinephrine was later confirmed by isotope experiments (Udenfriend and Wyngaarden, 1956). All the enzymes involved in the biosynthesis of catecholamines had been discovered and their regulatory mechanisms elucidated by 1964 (Udenfriend, 1966). Recent investigations in catecholamine biosynthesis suggest that epinine may be an important intermediate in the pathway (Laduron et al., 1974). This catecholamine was first proposed as the immediate precursor of epinephrine by Halle in 1906. Subsequently in vitro studies have shown the formation of epinine from dopamine by adrenal N-methyltransferase (Laduron, 1972) and the conversion of epinine into epinephrine by dopamine-β-hydroxylase (Bridgers and Kaufman, 1972).

The first step in the biosynthesis of epinephrine from tyrosine is the conversion of the amino acid into 3,4-dihydroxyphenylalanine (DOPA). The reaction is catalyzed by the enzyme tyrosine hydroxylase (TH), which was first isolated and characterized by Nagatsu et al. (1964). TH appears to be localized in the soluble fraction of bovine
adrenal chromaffin cells (Wurzburger and Musacchio, 1971). The natural cofactor of this enzyme is 6-methyltetrahydropterin (Lloyd et al., 1971). Activity of the enzyme requires molecular oxygen as a substrate (Daly et al., 1968), whereas the role of ferrous ion as a cofactor has not yet been established. Nevertheless, there are indications that ferrous ion not only stabilizes the enzyme but also stimulates the reaction (Nagatsu, 1973). The hydroxylation of tyrosine to DOPA in the guinea pig heart has been shown to be slower than all the subsequent steps in the biosynthetic pathway of epinephrine; therefore, this has been established to be the rate-limiting step (Levitt, 1965).

The second step in the biosynthesis of catecholamines involves the conversion of DOPA into dopamine and is catalyzed by the enzyme DOPA decarboxylase (DDC). This enzyme was first discovered by Holtz et al. in the mammalian kidney in 1938. Subsequent characterization of the enzyme indicates that it is not specific for DOPA, but decarboxylates a variety of aromatic L-amino acids (Lovenberg et al., 1962). DDC is widely distributed in a number of mammalian organs including adrenal glands, stomach and liver, and is present almost entirely in the soluble subcellular fraction (Blaschko et al., 1955). A number of observations suggest that pyridoxal-5-phosphate is the prosthetic group of this enzyme (Holtz and Bachman, 1952) and that it needs at least one free sulfhydryl group.
Dopamine-β-hydroxylase (DBH) is the enzyme responsible for the conversion of dopamine into norepinephrine. It was first isolated and characterized from adrenal medullary extracts (Levin et al., 1960) and subsequently purified to a single homogeneous protein (Friedman and Kaufman, 1965). Approximately one-half of the enzyme is membrane-bound in the adrenal chromaffin cell, while the other half is localized in the soluble fraction (Axelrod, 1973). DBH is released along with catecholamines during vesicular secretion (Kirshner, 1974). The enzyme contains copper and requires ascorbic acid as cofactor in this particular step (Kaufman, 1966). Enzyme activity is stimulated by the presence of dicarboxylic acids (Goldstein et al., 1965). DBH is also implicated in the β-hydroxylation of epinine, but it does not require ascorbic acid as a factor in this reaction (Bridgers and Kaufman, 1962). Recently, this enzyme was found to have a 24-hour circadian rhythm in rat adrenal glands and that this could be abolished by pinealectomy (Banerji and Quay, 1976).

The last step in the biosynthesis of epinephrine is catalyzed by the enzyme phenethanolamine-N-methyltransferase (PNMT). The activity of this enzyme converting norepinephrine into epinephrine was first described by Bülbbring (1949). The enzyme later was partially purified
and characterized by Axelrod (1962), who found that S-adenosyl methionine was the required cofactor and that PNMT was dependent on intact sulfhydryl groups for activity. The enzyme also shows high specificity towards phenethanolamines and is highly localized in the soluble fraction of the adrenal chromaffin tissues (Axelrod, 1966). A non-specific N-methyltransferase was proposed to be the enzyme responsible for the methylation of dopamine to epinine (Laduron, 1972).

Regulation of catecholamine biosynthesis comprises a number of mechanisms which can be divided into two categories: short- and long-term. Short-term regulation occurs in the cytosol of the chromaffin cell where feedback inhibition is exerted by catecholamines on tyrosine hydroxylase, the proposed rate-limiting enzyme. There is evidence for the existence of a small, particle-free, cytoplasmic pool of catecholamines (Alousi and Weiner, 1966) which may exert their feedback action by competing with the tetrahydrobiopterin cofactor of the enzyme (Nagatsu et al., 1972). Other potential sites of regulation include the membrane of the chromaffin vesicle as this structure regulates the access of dopamine into the vesicle to be hydroxylated (Molinoff et al., 1974). Endogenous inhibition of DBH has been identified in rat adrenal glands and may limit the rate of dopamine hydroxylation in intact tissues (Orcutt and Molinoff, 1976).

Long-term regulation is controlled by affecting the
formation of new enzymes. This can be achieved in a number of ways. It has been observed in recent years that the catecholamine biosynthetic enzymes can be induced to increase in activity after catecholamine depletion, which led to the concept of transsynaptic induction. There is considerable evidence that tyrosine hydroxylase, dopamine-β-hydroxylase and phenethanolamine-N-methyltransferase are partially controlled by this type of regulation (Mueller et al., 1969a; Thoenen et al., 1970; Ciaranello and Black, 1971). Acetylcholine is believed to be the immediate mediator of transsynaptic induction, based on the fact that ganglionic blocking agents abolish tyrosine hydroxylase induction (Mueller et al., 1970) whereas carbachol can mimic the induction effect (Guidotti and Costa, 1973). The attachment of acetylcholine to a receptor on the postsynaptic nerve cell membrane after its release from a presynaptic nerve ending induces a specific subcellular effect. Cyclic adenosine 3',5'-monophosphate (cAMP) has been proposed as a mediator between the receptor and the synthetic machinery for the catecholamine biosynthetic enzymes (Guidotti et al., 1973). However, the involvement of cyclic nucleotide in the induction of these enzymes is controversial at present, since such induction has been shown to occur without a preceding increase in cAMP (Otten et al., 1973). Protein synthesis is implicated in transsynaptic induction as the entire process can be abolished if actinomycin is
administered immediately after catecholamine depletion by reserpine (Mueller et al., 1969b).

Adrenal cortical hormones also play an important role in the long-term regulation. The enzyme most significantly influenced by these hormones is PNMT. Coupland (1953) observed that norepinephrine was the main amine in those species of animals where the chromaffin cells were not in contact with adrenal cortical tissues. He proposed that the corticosteroids were important in the methylation of norepinephrine and this proposal has been proven to be correct. PNMT levels are markedly reduced by hypophysectomy but can be restored by the administration of adrenal cortical trophic hormone (ACTH) or glucocorticoids (Wurtman and Axelrod, 1966). Hypophysectomy also causes small but significant changes in tyrosine hydroxylase and dopamine-β-hydroxylase. The administration of ACTH can restore the activity of these enzymes (Wurtman et al., 1972), and recently glucocorticoids have been reported to be effective as well (Axelrod, 1975). Actinomycin D or puromycin can block the increase in PNMT activity induced in vivo by dexamethasone. This suggests that corticosteroids increase enzyme activity by inducing their synthesis (Wurtman et al., 1972).

Catecholamine Secretion. Very little is known at present about the molecular events that couple stimulation to secretion in the adrenal medulla. Both nicotinic and
Muscarinic receptors were identified in the adrenal medulla and acetylcholine has been shown to be the normal physiological mediator of excitation of the adrenal medulla (Feldberg and Minz, 1934). The release of neurotransmitters from the terminal varicosities of a neuron initiated by the arrival of action potentials is called the stimulus-secretion coupling. The process is calcium dependent (Douglas, 1968). It is not known how this divalent cation exerts its effect in the chromaffin cell (Stjärne, 1972). There are two schools of thought as to how the vesicles travel from the cytoplasm to the plasma membrane of the chromaffin cell:

1. Random displacement by Brownian movement carries the vesicles to the membrane, provided the viscosity of the cytoplasm is not unusually high (Matthews, 1970).

2. The vesicles are transported by microtubules to the plasma membrane. Support for this theory includes the observation that colchicine, a drug which destroys the microtubules, inhibits the release of norepinephrine and DBH from the adrenal medulla (Douglas and Sorimachi, 1972).

Small amounts of lysolecithin can bring about the fusion of cell membranes (Lucy, 1970). Since this phospholipid comprises 17% of the adrenal vesicles (Lagercrantz, 1971), it is highly probable that this compound is involved
in the attachment of the vesicle to the plasma membrane. Low concentrations of ATP and magnesium may in certain media accelerate the spontaneous release of catecholamines as well as that of ATP and protein (Poisner and Trifaro, 1967). This has led to the proposition that the ATP-Mg may be involved in a step of the stimulated secretion of the vesicles from chromaffin cells (Stjarne, 1972). Lysolecithin may also be involved directly in the secretory process by inflicting local damage to the vesicular membrane (Blaschko et al., 1967; Douglas, 1968). The process of exocytosis has been proposed as a mechanism for the release of catecholamines on the basis of electron microscopic studies (De Robertis and Sabatini, 1960). Later, unequivocal microscopic evidence of exocytosis in the adrenal medulla of the hamster was reported (Diner, 1967). The current consensus is that exocytosis is an all-or-none phenomenon, with the vesicle releasing its entire soluble content to the exterior of the cell (Slotkin and Kirshner, 1973). It appears that the membrane of the secretory vesicles after exocytosis does not remain fused to the plasma membrane of the chromaffin cell, but is specifically retrieved (Benedeczky and Smith, 1972). The process of retrieval involves the formation of a bristle-coating on the cytoplasmic face of the vesicular membrane, followed by the fission of the membrane into several smaller vesicles as it separates from the plasma membrane (Smith, 1973).
Catecholamine Uptake and Storage. Reuptake is an important route of inactivation of catecholamines after their release. Some investigators claim that more than 90% of the released transmitters are recaptured (Haefely et al., 1964). The neuronal uptake of norepinephrine (Uptake₁) is mediated by a high affinity and a stereochemically selective transport system located in the axonal membrane of the adrenergic neuron (Iversen, 1971). This system is highly dependent on the presence of sodium ions and is blocked by cocaine and tricyclic anti-depressants. In addition, a second uptake system (Uptake₂) is present in various glandular and smooth-muscle tissues (Gillespie, 1973). This system is membrane-bound but differs from Uptake₁ in having a lower affinity for norepinephrine and a higher affinity for N-substituted catecholamines such as epinephrine (Iversen, 1974). The catecholamines are taken up by storage vesicles after having been transported through the neuronal membrane.

Early studies of the distribution of tracer amounts of radioactive-labelled catecholamines have indicated that the adrenal medulla is highly efficient in accumulating the labelled material (Axelrod et al., 1959; Whitby et al., 1961). This led to later studies which demonstrated the existence of a catecholamine uptake system in isolated adrenal medullary vesicles (Kirshner, 1962; Carlsson et al., 1962). Numerous experiments have been done since then to
characterize this system. It has been found to be tem-
perature-sensitive: the incorporation of amines at 37°C
is twice that at 30°C. It is magnesium-ATP dependent as
the presence of these substances enhance the uptake
several fold. The system is inhibited by reserpine and
certain other drugs including sulfhydryl agents such as
N-ethyImaleimide and chelating agents such as EDTA
(Kirshner, 1974). The uptake process is quite specific.
Serotonin is preferred over catecholamines which, in turn,
are preferred over related phenethylamines (Slotkin, 1973a).
The process can be saturated. Uptake of epinephrine in
bovine adrenal vesicles has an estimated $K_m$ of $4-8 \times 10^{-4}$M
(Lundborg, 1966).

Recent advances in the knowledge of the uptake sys-
tem allows one to regard uptake and storage as two distinct
processes. Slotkin (1971) observed that serotonin was
incorporated to a greater extent than epinephrine despite
the fact that it was not stored as stably as epinephrine.
It was also observed that although competition occurred
among related amines for the same uptake system, their com-
petitive potency was unrelated to subsequent storage
stability (Slotkin and Kirshner, 1971). A model proposed
by Slotkin (1974) suggests the attachment of a catecholamine
molecule to a carrier at the outside surface of the vesicu-
lar membrane. The amine-carrier complex subsequently dis-
sociates on the inside surface by transphosphorylation with
ATP.
Some amines such as metaraminol may be taken up by a second uptake system (Lundborg, 1966). This system is independent of magnesium and ATP and is relatively insensitive to the inhibition by reserpine in vitro (Lundborg and Stitzel, 1967). Metaraminol appears to be preferred over tyramine which is preferred over epinephrine (Slotkin and Kirshner, 1971). *In vivo* experiments, however, have demonstrated that norepinephrine is concentrated and stored by this second system after the Mg-ATP system has been blocked by reserpine (Lundborg and Stitzel, 1967). Amine efflux from bovine vesicles occurs at different rates with the two different uptake systems. Typical efflux curves obtained with metaraminol are bi-phasic, with a rapid and a slow component. Seventy five percent of the labeled metaraminol is released with a half-time of about 5 minutes and 25% with a half-time of about 42 minutes. Epinephrine has a different efflux pattern as 22% of the amine is released with a half-time of about 5 minutes and 78% with a half-time of about 160 minutes (Slotkin and Kirshner, 1971). Therefore, one can conclude that metaraminol is distributed primarily into the fast pool while epinephrine is distributed primarily into the slow pool. The two pools may reflect the existence of two populations of storage vesicles or two different pools within the same vesicle which do not exchange amines with each other (Slotkin et al., 1971). It has been observed that reserpine does not alter the half-times of
either the fast or the slow pool, indicating that this drug exerts its effect through the blockade of the uptake process rather than by disrupting the storage complex (Slotkin et al., 1971).

Epinephrine is believed to be bound to a matrix composed of ATP and chromogranins after entry into the vesicle (Kirshner, 1974). Calcium may also be part of the binding complex (Borowitz, 1970). Physical chemical studies indicate that these components interact with each other through weak intermolecular forces which are rapidly made and broken. A complex is thus formed as a result of the statistical kinetics of these interactions (Kirshner, 1974).

Enzymatic Catabolism. The catabolism of catecholamines generally involves two different enzymes: a monoamine oxidase (MAO), which deaminates the catecholamines to form aldehydes and a catechol-O-methyltransferase (COMT) which transfers methyl groups to the metahydroxyl groups of the catecholamines.

Monoamine oxidase was first discovered as tyramine oxidase in the liver (Hare, 1928). It was subsequently observed to be present in many tissues with the catecholamines being among the many monoamines serving as its substrates (Blaschko et al., 1937). In the adrenergic neuron, the enzyme was shown to be localized exclusively in the outer membrane of the mitochondria (Tipton, 1973). MAO is also widely distributed extraneuronally. The liver, parotid and
submaxillary glands are known to have the highest activity (Nagatsu, 1973). The enzyme contains a flavin as a prosthetic group which is involved in the oxidation of the substrate (Igaue et al., 1967). Deamination of epinephrine or norepinephrine by MAO results in the formation of 3,4-dihydroxymandelicaldehyde which may undergo further oxidation to 3,4-dihydroxyphenylglycol (Kopin, 1972). Recently, the enzyme has been demonstrated to exist in multiple forms (Youdim et al., 1969) although the significance of these forms remains to be elucidated.

The enzymatic O-methylation of catecholamines was first discovered by Axelrod (1957) who later characterized and named the enzyme to be catechol-O-methyltransferase (COMT) (Axelrod and Tomchick, 1958). This enzyme is localized both intra- and extraneuronally. The intraneuronal enzyme is found mainly in the soluble fraction of the sympathetic nerve terminals (Broch and Fonnum, 1972). Extraneuronal COMT is found in a variety of tissues including the liver (Inscoe et al., 1965). The enzyme is present in the soluble as well as the particulate fractions of these extraneuronal tissues (Verity et al., 1972). It inactivates catecholamines by transferring the methyl group from S-adenosylmethionine to a wide variety of catechols including those which have been deaminated (Axelrod, 1959).

COMT appears to be the major enzymatic means for terminating the effects of catecholamines extraneuronally but MAO provides an important alternative route of metabolic
inactivation (Kopin, 1972). The two enzymes are localized in Uptake₂ sites and are important for the degradation of catecholamines there (Iversen, 1974).

The termination of the action of endogenous and exogenous catecholamines is mainly through physical inactivation via either reuptake into the neuron (Uptake₁) or uptake into extraneuronal sites (Uptake₂). COMT and MAO play secondary roles as inhibition of these enzymes did not potentiate cardiovascular responses initiated by exogenously administered catecholamines (Crout, 1961).

Ontogenesis of the Sympathetic Nervous System

The primitive cellular precursors of the sympathetic nervous system are believed to arise from ectodermal elements in the neural crest region of the embryo (Coupland, 1965b). These stem cells subsequently differentiate into neuroblasts and pheochromoblasts. During development, the stem cells of the neuroblasts migrate to peripheral tissues and form the paravertebral and preaortic sympathetic ganglia, from which the postganglionic sympathetic neurons finally emerge. Those stem cells which are destined to become pheochromocytes either give rise to extra-adrenal chromaffin cells or they invade the adrenal cortex to form the primordial adrenal medulla (Levine and Landsberg, 1974).

The sympathetic nervous system matures both morphologically and functionally. Fluorescent microscopic studies indicate that the mature neuron contains a larger
number of varicosities with higher concentrations of catecholamines than the immature ones (Schiebler and Heene, 1968). The presence of catecholamines has been demonstrated during rat development as early as the fertilized ovum stage prior to and during cleavage (Burden and Lawrence, 1973). Generally, the content of catecholamines of most adrenergically innervated mammalian tissues is low during fetal and early postnatal life (Iversen, 1967), though there is some species variation. Several phylogenetic studies have demonstrated a correlation between the development of the heart and increments in catecholamine concentration in the cardiac tissue (Mirkin, 1972). The unique exception is the calf heart, which contains the same concentration of norepinephrine as the adult bovine heart (Vogel et al., 1969).

The appearance of the catecholamine biosynthetic enzymes represent the development of the functional aspect of the sympathetic nervous system. Tyrosine hydroxylase was the first to be documented using in vivo studies in the chick embryo (Ignarro and Shideman, 1968). The increase of DBH was correlated to the development of central adrenergic neurons (Coyle and Axelrod, 1972). The subcellular distribution of these enzymes shifts from the cell bodies of the neurons to the nerve terminals during maturation (Coyle, 1973).

The activity of the catabolic enzymes also appears to increase with development. MAO and COMT initially were
detected in the embryonic chick on the fourth day of incubation and reached maximal levels by the nineteenth day (Ignarro and Shideman, 1968). The activities of these two enzymes in swine kidney and liver increased with age. The development was more variable in other tissues (Stanton et al., 1975).

The development of the uptake capability of the sympathetic neuron generally parallels the appearance of endogenous catecholamines in the innervated tissue. Excellent correlation between these two events has been noted in the heart and spleen (Iversen et al., 1967). The storage of catecholamines follows a similar pattern. Catecholamine storage is preceded by the storage of ATP during development of the adrenal medulla. Biochemical analysis of the adrenal medulla in rabbits and rats during pre- and postnatal storages has indicated that the molar ratio of catecholamines to ATP increases many fold from the seventeenth day after conception to adult age (O'Brien et al., 1972). This suggests that the fetal adrenal medulla contains vesicles which mainly store ATP and which gradually take up catecholamines during the course of maturation. Microscopic findings confirm these biochemical results (Pletscher et al., 1973). The vesicles of the rat are denser at birth than in older animals. It has been suggested that the rate of synthesis of the storage vesicles is low at birth but increases rapidly during maturation (Slotkin, 1973b).
Epinephrine and norepinephrine have both been observed to be present in the adrenal medulla of the fetal rabbit (Brundin, 1965). Epinephrine- and norepinephrine-containing cells are found in the fetal swine adrenal medulla at about nine weeks of age (Podgornaya, 1968).

It is also of interest to note that transsynaptic enzyme induction appears to play an important part in the development of the sympathetic nervous system. Denervation of the adrenal medulla prevents age-dependent increases in enzyme activity and catecholamine content (Patrick and Kirshner, 1972). The appearance of transsynaptic enzyme induction seems to reflect the maturation of neuronal tissues of the rat. Toxic doses of reserpine induced tyrosine hydroxylase in the adult rat but did not do so in neonates during the first three weeks of life (Black and Reis, 1975).

**Perinatal Survival of the Swine**

The large number of stressors the perinatal piglet encounters may be partially responsible for the high mortality rate. The swine is a polytocous species; therefore, the fetuses face nutritional competition *in utero* (Pomeroy, 1960). The survival of the fetus is also dependent on its position in the uterus because of the differential transport of nutrients and gases to different parts of the uterus (Dawes, 1968). The maternal environment, often influenced by external factors, may play an important part
in determining the viability of the fetus (Stanton and Carroll, 1974). Asphyxia due to reduced respiratory-gas exchange between the fetus and the placenta during parturition imposes a severe survival problem (James et al., 1958). Prenatal asphyxia is not only associated with prenatal death, but with postnatal morbidity as well (Curtis, 1974).

The pig also faces competition for food with its litter mates during the neonatal period. In addition, it is no longer sheltered in the uterus and is exposed to many hazards such as chilling, infection, crushing and trampling by the sow.

Chilling is particularly insidious and is a major cause of neonatal death. It may not only cause death directly, but chilling also predisposes the neonates to crushing, starvation, and disease since the chilled piglet is lethargic and may not nurse or seek the sow. The newborn piglet is highly susceptible to cold, with a thermoneutral temperature of 32 - 35°C. This is high compared to many other species including the lamb and the calf which have thermoneutral temperatures of about 29°C and 13°C, respectively (Curtis, 1974). The marked intolerance to cold in the neonatal piglet is partially due to the large surface area and the sparse pelage of the animal (Mount, 1968). The absence of brown fat deprives the piglet of one of the important heat generating mechanisms—nonshivering thermogenesis (Brück, 1970). The total fat
content of the pig constitutes only about 1% of the body weight at birth (Widdowson, 1950); therefore, the insulative capacity is minimal in a pig of this age.

The immaturity of the immune system in the neonatal piglet predisposes the animal to infections. Measurable developments in the immune system do not occur until the piglet has reached 10 to 14 days of age. The neonate is completely dependent before this time on the colostrum supplied by its mother which contains the necessary immunoglobulins (Wilson, 1974).

Neonatal piglets are deficient in their capability for the \( \beta \)-oxidation of fatty acids. This may be related to the low number of mitochondria present in the liver for the first 48 hours of life (Mersmann, 1974). Hepatic gluconeogenesis also occurs at a much-lower rate than with older ages.

The cardiovascular system of the neonatal piglet is quite immature. The heart rate of these animals is highly susceptible to changes in blood glucose concentration. Hypoglycemia is associated with a rapid fall of heart rate, a decrease in body temperature and frequently death. Newborn piglets have a heart rate of 200-300/minute which decreases in rate exponentially with growth (Engelhardt, 1966). Blood hemoglobin levels in pigs under 6 weeks of age is highly sensitive to inadequate dietary iron (Bustad, 1966) and parenteral iron frequently must be provided.
What is the role of the sympathetic nervous system of a perinatal piglet exposed to stressors? Prenatal and intrapartum anoxia have been shown to directly activate the adrenosympathetic system of the bovine fetus (Comline and Silver, 1966b). However, the catecholamines released may also exert several actions harmful to the neonate. Epinephrine and norepinephrine infusions given to newborn piglets have been shown to decrease blood volume and increase interstitial volume (Cheek and Rowe, 1966). Mobilization of carbohydrate stores during parturition may reduce the physiological reserves of the newborn and seriously hamper the animal's ability to withstand further stress (Stanton and Carroll, 1974). The piglet crouches and shivers violently in cold but cannot generate heat by nonshivering metabolic means. Although the sympathetic system does not seem to be directly involved in shivering, it plays an important role in reducing heat loss by inducing peripheral vasoconstriction (Stanton and Mueller, 1973a). Catecholamine-induced mobilization of lipids, though less important for cold adaptation of the newborn pigs, is part of a thermoregulatory response to cold in pigs older than 50 hours (Mersmann, 1974).

The neonatal piglet responds to starvation by mobilizing liver glycogen stores (Stanton and Mueller, 1974). It may also be very dependent on glycolysis as indicated by increased plasma lactate levels during starvation (Stanton et al., 1973).
Thus, activation of the sympathetic nervous system constitutes part of the adaptive response of the neonatal piglet to many of the environmental stressors. Any dyssynchrony in the system should result in a lowered capability of the animal to withstand stress.
Chapter II

MATERIALS AND METHODS

Animals

The crossbred swine (Hampshire x Yorkshire x Duroc) used in these studies were born and reared in temperature-controlled facilities and handled according to standard swine husbandry practices at the Shell Development Company Biological Sciences Research Center in Modesto, California. The animals were transported in temperature-controlled vehicles a short distance from the barns to the laboratory and were kept near thermoneutrality (33 - 35°C for newborn and 27°C for older animals). Food and water were given ad libitum. The diets of the pig varied with age and are presented in Table II. The newborn and 14-day-old pigs were stunned with a blow on the head by a hammer while the 70-day-old animals were stunned with a captive-bolt pistol. The animals were exsanguinated and the adrenal glands quickly removed and placed on ice.

Estimation of Medullary and Cortical Volumes

Adrenal glands were rapidly removed from the animals
TABLE II
Diet Compositions, Percent

<table>
<thead>
<tr>
<th>Component</th>
<th>Sow, Gestation</th>
<th>Sow, Lactation</th>
<th>Baby Pig, 21% Protein (0-34 days)</th>
<th>Grower, 18% Protein (35-60 days)</th>
<th>Finisher, 15% Protein (60 days-adult)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Milo</td>
<td>--</td>
<td>--</td>
<td>14.75</td>
<td>1.66</td>
<td>1.66</td>
</tr>
<tr>
<td>Corn grain</td>
<td>77.42</td>
<td>72.50</td>
<td>46.11</td>
<td>71.67</td>
<td>78.86</td>
</tr>
<tr>
<td>Soybean oil meal</td>
<td>7.85</td>
<td>9.62</td>
<td>20.00</td>
<td>15.00</td>
<td>4.00</td>
</tr>
<tr>
<td>Bran</td>
<td>2.80</td>
<td>3.46</td>
<td>--</td>
<td>9.50</td>
<td>--</td>
</tr>
<tr>
<td>Alfalfa meal</td>
<td>3.90</td>
<td>4.79</td>
<td>2.03</td>
<td>5.00</td>
<td>4.00</td>
</tr>
<tr>
<td>Cottonseed meal</td>
<td>--</td>
<td>--</td>
<td>2.93</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Meat and bone meal</td>
<td>5.60</td>
<td>6.88</td>
<td>5.00</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Fish soluble</td>
<td>0.60</td>
<td>0.68</td>
<td>2.67</td>
<td>5.00</td>
<td>4.00</td>
</tr>
<tr>
<td>Whey (delactose)</td>
<td>--</td>
<td>--</td>
<td>6.67</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Salt (iodized)</td>
<td>0.60</td>
<td>0.68</td>
<td>0.40</td>
<td>0.50</td>
<td>0.50</td>
</tr>
<tr>
<td>Vitamin premix</td>
<td>0.60</td>
<td>0.68</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Limestone</td>
<td>0.60</td>
<td>0.68</td>
<td>--</td>
<td>0.70</td>
<td>0.90</td>
</tr>
<tr>
<td>Trace mineral supplement</td>
<td>0.03</td>
<td>0.03</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Vitamin and mineral premix</td>
<td>--</td>
<td>--</td>
<td>0.20</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Vitamin and mineral premix</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>0.17</td>
<td>0.16</td>
</tr>
<tr>
<td>Dicalcium phosphate</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>0.30</td>
<td>0.40</td>
</tr>
<tr>
<td>D., L. methionine</td>
<td>--</td>
<td>--</td>
<td>0.04</td>
<td>--</td>
<td>--</td>
</tr>
</tbody>
</table>

Modified from Siers et al., 1976.
and immersed in formaldehyde for three days. The left gland of each pair was divided longitudinally into three or four sections, depending upon the size of the gland. Four centimeter-thick sections were embedded in paraffin blocks and cut into five or six thinner slices. These slices were subsequently treated with hematoxylin-eosin stain and mounted on glass (slides prepared by Miller Clinical Laboratory, Modesto, California). The areas of the medulla and the cortex were estimated from representative slices of each section with a grid. The slices were enlarged twenty times with a Nikon Profile projector (Nikon Inc., Instrument Division, Garden City, New York), and the grid was attached to the screen. Volumes of the medulla and the cortex were calculated by integrating the areas estimated from the slices over the whole length of the adrenal gland.

**Determination of Catecholamines**

The method of this determination was modified from the procedure of Anton and Sayer (1962). Adrenal glands were trimmed, weighed and homogenized in ice cold 0.4 N perchloric acid (PCA) in a concentration of 100 mg per 4 ml using a Brinkman Polytron homogenizer. The homogenates were centrifuged at 40,000 g for 20 minutes in a Sorvall RC2-B refrigerated centrifuge. The supernatants were diluted to 2.5 mg/ml with 0.4 N PCA and transferred to 50-ml beakers. Norepinephrine and epinephrine standards
contained 1 ml (5 μg/ml) each of the respective catecholamine. The blank contained 1 ml of 0.4 N PCA; 4 ml of water and 15 ml of 0.1% EDTA were subsequently added to each beaker. The solutions were adjusted to pH 6.5 with 0.5 M K₂CO₃ and placed on Bio-Rex 70 carboxylic acid resin columns (BioRad Lab., Richmond, California). The columns were washed with 20 ml of water and the catecholamines were eluted with 5 ml of 0.5 N HCl. Two separate aliquots of 0.6 ml from each sample were used for the analysis. One set of the aliquots was used for the measurement of norepinephrine at pH 6.5 and the other set for the measurement of epinephrine at pH 2.4. For norepinephrine, 0.1 ml of 1 M phosphate buffer and 0.2 ml of 0.15 N NaOH were added to each sample immediately before the assay. The samples were then oxidized for exactly one minute with 0.05 ml of 0.3% K₃Fe(CN)₆. The oxidation was stopped with 0.6 ml of alkaline ascorbate (10% ascorbic acid in 5 ml of 10 N NaOH prepared fresh before each assay). One and five tenths ml of water was added between 15 and 30 seconds afterwards. Fluorescence was recorded at exactly three minutes with an Aminco-Bowman spectrofluorometer. The excitation and emission wavelengths were 395 and 506 μm, respectively. The samples and the epinephrine standard were read against the relative fluorescence setting of the norepinephrine standard at 100 and the blank at 0. The measurement of epinephrine was similar to that described for norepinephrine. However, 0.3 ml of water was substituted for the phosphate.
buffer and sodium hydroxide before the assay to keep the pH at 2.4. The excitation and emission wavelengths were 414 and 522 nm, respectively. The samples and norepinephrine standard were read against a setting of epinephrine standard at 100 and the blank at 0. Standard curves for the catecholamines are shown in Figures 1 and 2.

**Determination of Catecholamine Biosynthetic Enzymes Activities**

The adrenal glands were trimmed and homogenized in ice-cold isotonic 0.15 M KCl using a Brinkman Polytron homogenizer. The homogenates were used for the analysis of the catecholamine biosynthetic enzymes. Before using, the homogenates were dialyzed overnight at 4°C against 100 volumes of 5 mM Tris-Cl buffer at pH 7.0 to remove catecholamines and other endogenous inhibitors of small molecular weight. The dialysis did not change the activity of either DOPA decarboxylase or phenethanolamine-N-methyltransferase.

Two methods of DOPA decarboxylase assay were used. The first method was not satisfactory because we were unable to saturate the enzyme. The results reported in Table VI were assayed by this method. Data from the two DOPA decarboxylase methods correlate very well with each other.

In the assay for Dopamine-ß-hydroxylase, N-ethylmaleimide did not block the activity of endogenous
Figure 1. Norepinephrine standard curve.

Figure 2. Epinephrine standard curve.
inhibitors in swine adrenal tissues, but dialysis removed their inhibition. Copper was added in the incubation mixture to replace that which was removed by dialysis.

The protein of the adrenal tissues was determined by the Biuret method.

**Tyrosine Hydroxylase (TH)**

The assay method was modified from the procedure of Nagatsu et al. (1964). Figure 3 shows the determination of optimal amount of homogenate, time of incubation and substrate concentration for this enzyme. The incubation mixture for the enzyme assay contained: 0.1 ml of 1 M Tris-maleate buffer (pH 5.7), 0.1 ml of 5 mM Fe(NH$_4$)$_2$($SO_4$)$_2$, 0.1 ml of 10 mM 6,7-dimethyl-5,6,7,8-tetrahydropterine (DMPH$_4$) dissolved in 1 M mercaptoethanol, 0.4 ml of tissue homogenate, 0.1 ml of 1 mM L-tyrosine containing 10-20 μCi/ml of 3,5-$^3$H-tyrosine and 0.1 ml of glass-distilled water. The mixture was incubated for exactly 10 minutes at 37°C with constant shaking. The reaction was stopped by adding 0.1 ml of 30% trichloroacetic acid and the precipitated protein was removed by centrifugation of the sample at 1000 g for 15 minutes. Five-tenths ml of the supernatant followed by 1 ml of water was placed on a 5 x 30 mm Dowex 50 W-X$_4$ H$^+$ column (100-200 mesh). The radioactivity of 0.5 ml of the eluate was determined in a liquid scintillation spectrometer using 15 ml of Bray's scintillation medium.
Figure 3. Determination of optimal amount of homogenate, incubation time, and substrate concentration for the assay of tyrosine hydroxylase.
DOPA Decarboxylase (DDC): Method 1

This method was modified from the procedure of Creveling and Daly (1971a). The incubation mixture contained: 0.3 ml of 1.08 M NaH2PO4, 0.23 M Na2B4O7 buffer (pH 6.9), 0.025 ml of 3.2 mM pyridoxal 5'-phosphate, 0.025 ml of 0.03 M tranylcypromine sulfate, 0.15 ml of glass distilled water, 0.2 ml of tissue homogenate, 0.05 ml of 0.4 mM 3,4-dihydroxy L-phenylalanine (L-DOPA) containing 10 μCi/ml of D,L-14C DOPA. The mixture was incubated for exactly 20 minutes at 37°C with constant shaking. The reaction was stopped by chilling the sample to 0°C in an ice bath. Ten ml of acid-free butanol was added and the mixture shaken for 5 minutes followed by centrifuging at 100 g for 10 minutes. One ml of the upper organic layer was added to 10 ml of Bray's medium for the determination of radioactivity.

DOPA Decarboxylase (DDC): Method 2

This assay was modified from the procedure of McCaman et al. (1972). Figure 4 shows the optimal determination of amount of homogenate, time of incubation and substrate concentration for this enzyme. The incubation mixture contained: 0.05 ml of premixed buffer containing 0.4 M NaH2PO4(pH 7.0), 4 mM tranylcypromine sulphate and 0.4 mM pyridoxal phosphate; 0.05 ml glass-distilled water, 0.5 ml of homogenate and 0.05 ml of 0.4 mM 3,4-dihydroxy L-phenylalanine (L-DOPA) containing 10 μCi/ml.
Figure 4. Determination of optional amount of homogenate, incubation time and substrate concentration for the assay of DOPA decarboxylase.
of D,L-\textsuperscript{14}C DOPA. The mixture was incubated for exactly 30 minutes at 37°C with constant shaking. To stop the reaction, 0.2 ml of ice cold 0.05 M NaH\textsubscript{2}PO\textsubscript{4}(pH 7.0) followed by 0.2 ml of glass-distilled water was added. Dopamine, the reaction product, was extracted by adding 0.2 ml of 0.1 M bisdiethylhexylphosphoric acid (DEHPA) in CHCl\textsubscript{3}. The mixture was vortexed for 15 seconds and centrifuged at 1000 g for 10 minutes. The top aqueous layer was aspirated and discarded. An additional 0.2 ml of 0.05 M NaH\textsubscript{2}PO\textsubscript{4} was added and the separation procedure repeated. One tenth ml of the lower organic phase was subsequently dissolved in 1 ml of 0.3 M hyamine in methanol. The solution was added to 15 ml of Bray's scintillation medium for the determination of radioactivity.

**Dopamine-\textsuperscript{\beta}-hydroxylase (DBH)**

This method was modified from the procedure of Creveling and Daly (1971b). Figure 5 shows the optimal determination of amount of homogenate, time of incubation and substrate concentration for this enzyme. The incubation mixture contained: 0.2 ml of 1 M KH\textsubscript{2}PO\textsubscript{4} buffer (pH 5.5), 0.05 ml of 0.2 M fumarate, 0.05 ml of 0.2 M ascorbate, 0.05 ml of 0.1 M tranylcypromine sulfate, 0.05 ml of 0.4 mM cupric sulfate, 0.05 ml of catalase (75 mg/ml) 0.15 ml of glass-distilled water, 0.2 ml of tissue homogenate, 0.2 ml of 1 mM tyramine containing 10 \mu Ci/ml of \textsuperscript{3}H-tyramine. The mixture was incubated for
Figure 5. Determination of optimal amount of homogenate, incubation time and substrate concentration for the assay of dopamine-β-hydroxylase.
exactly 15 minutes at 37°C with constant shaking. The re-
action was stopped with the addition of 1 ml of 7% per-
chloric acid. The precipitated protein was removed after
the sample was centrifuged at 1000 g for 10 minutes. One
ml of the supernatant was mixed with 0.3 ml of 12.45 N
ammonium hydroxide and 0.3 ml of 2% sodium periodate. The
mixture was incubated for exactly 4 minutes at room tem-
perature. Three tenths ml of 10% NaHSO₃ and 1 ml of 5 N
HCl were added and 1 ml of the mixture was removed and
placed in screw-capped culture tubes with teflon caps
(16 x 100 mm) along with 5 ml of toluene. The mixture was
centrifuged at 1000 g for 10 minutes. Four ml of the top
organic layer was delivered to the counting vial along
with 10 ml of 0.56% omnifluor-toluene solution for the
determination of radioactivity.

Phenethanolamine-N-Methyltransferase (PNMT)

This method was modified from the procedure of
Creveling and Daly (1971). The dialyzed homogenate was
centrifuged at 40,000 g for exactly 1 hour in a Sorvall
RC2-B refrigerated centrifuge before incubation. Figure
6 shows the optimal determination of amount of homogenate,
time of incubation and substrate concentration for this
enzyme. The incubation mixture was placed in screw-capped
culture tubes (16 x 100 mm) with Teflon-lined caps and
contained: 0.1 ml of 0.5 M Tris buffer (pH 8.5), 0.1 ml
of 0.15 M KCl, 0.2 ml of the supernatant, 0.05 ml of 0.4 mM
Figure 6. Determination of optional amount of homogenate, incubation time and substrate concentration for the assay of phenethanolamine-N-methyltransferase.
S-adenosylmethionine containing 1 μCi/ml of \(^{14}\)C S-adenosylmethionine, 0.05 ml of 30 mM phenethanolamine. The mixture was incubated for exactly 30 minutes at 37°C with constant shaking. The reaction was stopped by adding 0.5 ml of 0.5 M sodium borate buffer (pH 10) followed by 6 ml of toluene-isoamyl alcohol (30:1). The tubes were then capped and shaken vigorously. The organic layers were separated by centrifuging at 1000 g for 10 minutes. Four ml of the top organic layer was added to 10 ml of 0.56% omnifluortoluene solution for the determination of radioactivity.

**Biuret Method**

The method was modified from the procedure of Gornall et al. (1949). The biuret reagent contained: 1.5 g of CuSO\(_4\) • 5H\(_2\)O, 6.0 g of NaK \(_C_4H_6O_6\) • 4H\(_2\)O (sodium potassium tartrate), 300 ml. of 10% NaOH and glass-distilled water was added to make 1 liter. The incubation mixture for the samples contained: 0.2 ml of homogenate, 1.1 ml of glass-distilled water and 0.2 ml of 10% deoxycholate (DOCA). The standard mixture contained: 1.0 ml of bovine serum albumin (1 mg/ml), 0.3 ml of glass-distilled water and 0.2 ml of 10% DOCA. The blank mixture contained: 1.3 ml of glass-distilled water and 0.2 ml of 10% DOCA. The sample, standard and blank mixtures were incubated for exactly 15 minutes at 37°C. One and five tenths ml of the biuret reagent was added to each mixture, which was again
incubated for 15 minutes at 37°C. Optical density for each mixture was read at 540 nm with a Gilford 350 N micro-sample spectrophotometer.

**Estimation of Maximal Velocity (Vmax) and Michaelis Constant (KM)**

The Vmax and Km for the enzyme kinetic curves were both estimated by the non-linear least-squares method (Cleland, 1967; Wilkinson, 1961). The calculations were performed on a Hewlett-Packard model 9830A programmable calculator (Hewlett Packard, Palo Alto, California).

**Determination of Tyrosine Concentrations in Adrenal Gland and Plasma**

**Tyrosine Extraction from Adrenal Glands**

Adrenal glands were homogenized in 0.4 N perchloric acid solution in a concentration of 100 mg/ml. The homogenate was centrifuged at 40,000 g for 20 minutes in a Sorvall RC2-B refrigerated centrifuge. Two ml of the supernatant was combined with 8 ml of 0.1% EDTA and the mixture was titrated to pH 6.5 with 2 N K₂CO₃. The solution was placed on a Bio-Rex 70 carboxylic acid resin column (BioRad Lab., Richmond, California) followed by 20 ml of glass-distilled water. The eluates were combined and titrated to pH 2.0 with 6 N HCl. This solution was placed on a BioRad AG5W-X8 resin column (100-200 mesh) followed by 20 ml of glass-distilled water and the eluates were discarded. Ten ml of 1 N NH₄OH
was added to the column and the eluate collected was dried overnight under a steady stream of air. The residue obtained was redissolved in 0.5 ml of 0.01 N HCl solution.

**Tyrosine Extraction from Plasma**

The method was modified from the procedure of Wong et al. (1964). The blood sample was centrifuged at 1000 g for 20 minutes. One volume of the plasma was added to an equal volume of 6 N trichloroacetic acid. The mixture was centrifuged at 1000 g for 5 minutes and the supernatant was saved for fluorometric determination.

**Fluorometric Determination of Tyrosine Concentration**

The method was modified from the procedure of Wong et al. (1964). The tyrosine extracts were assayed in mixtures each containing 0.1 ml of 1-nitroso-2-naphthol in 95% ethanol (3 mg/ml), 0.5 ml of an acid nitrite solution containing 3 mg of sodium nitrite per ml of 1.5 N nitric acid, 0.05 ml of the extract. The standard mixture contained 0.05 ml of L-tyrosine solution (0.025 mg/ml) instead of the extract. The mixture was incubated for exactly 30 minutes at 37°C with constant shaking. The reaction was stopped by adding 4 ml of glass-distilled water and 5 ml of ethylene dichloride. The resultant mixture was vigorously shaken for 5 minutes followed by 5 minutes of centrifugation of 1000 g. Four ml of the top aqueous
layer were transferred to a cuvette for fluorometric determination. Samples were read against a standard setting of 100 and blank setting of 0 in an Aminco-Bowman spectrofluorometer. The excitation and emission wavelengths were 460 and 570 mJ, respectively.

In Vitro Biosynthesis of Catecholamines from Tyrosine by Adrenal Slices

Adrenal glands were trimmed and sliced with a McIlwain mechanical tissue chopper (Brinkman Instruments, New York) to a thickness of 0.5 mm. Slices from both ends of the glands were discarded. A combination of slices from various sections of the gland weighing 200 mg was incubated in 2 ml of Krebs bicarbonate buffer. The buffer (pH 7.4) contained: 0.154 M NaCl, 0.154 M KCl, 0.055 M CaCl₂, 0.154 M KH₂PO₄, 0.154 M MgSO₄·7H₂O, 0.154 M NaHCO₃, 1 mg/ml glucose, 0.05 mM iproniazid, 0.4 mM of L-tyrosine containing 1 μ Ci of ¹⁴C-tyrosine or appropriate dilutions thereof. The buffer was saturated with 95% O₂ and 5% CO₂ just prior to the experiment. The tissue slices were incubated for exactly 2 hours at 37°C under a constant pressure of 95% O₂ and 5% CO₂. Duplicate controls were kept on ice. Incubation of the slices was stopped by drying them on tissue papers and immersing them in 2 ml of 0.4 N perchloric acid. They were homogenized by a Brinkman Polytron and centrifuged at 40,000 g for 20 minutes. The
supernatant was placed on Bio-Rex 70 carboxylic acid resin column and washed with 20 ml of water. Catecholamines were separated by the method of Minard and Grant (1972). They were eluted from the column in five continuous 5 ml fractions of 2% boric acid followed by five continuous 5 ml fractions of 0.2 N HCl. The elution pattern of norepinephrine and its O-methylated metabolite normetanephrine is shown in Figure 7. Five tenths ml of elute from each fraction was added to 15 ml of Bray's medium for the determination of radioactivity.

**Amine Uptake by Adrenal Vesicles**

The method was modified from the procedure of Slotkin (1973b). Adrenal glands were trimmed, weighed and placed in ice cold 0.3 M Sucrose-Hepes medium (pH 7.4). The glands were minced with scissors and homogenized in a Potter Elvehjem homogenizer at a concentration of 1 g/20 ml. Three ml of the crude homogenate were removed for tissue and vesicular catecholamine determination. The remainder of the crude homogenate was centrifuged at 800 g in a Sorvall RC2-B refrigerated centrifuge for 10 minutes. Figure 8 shows the optimal parameters for the experimental procedure. The incubation mixture contained: 0.6 ml of sucrose-Hepes buffer containing 0.3 M sucrose, 0.05 M Hepes, 0.05 mM iproniazid (pH 7.4); 0.1 ml of 0.05 M ATP-Mg, 0.2 ml of supernatant and 8 μg of epinephrine or metaraminol containing 1 μCi of 14C-epinephrine or 3H-metaraminol or
Figure 7. Elution pattern of norepinephrine and normethanephrine.

Norepinephrine: O--O

Normetanephrine: Δ- -Δ

E = sample eluate

W = 20 ml water eluate

B = 2% boric acid eluate in 5 continuous fractions of 5 ml each subscript denotes fraction number.

H = 0.2 N HCl eluate in 5 continuous fraction of 5 ml each.
Figure 8. Determination of optimal parameters of the amine uptake by isolated adrenal vesicles

Met = Metaraminol

Epi = Epinephrine

The parameters are obtained by one determination.
appropriate dilutions thereof. The suspension of vesicles was incubated for exactly 10 minutes at 37°C with constant shaking. Duplicate controls were kept on ice. Incubation was stopped by diluting the suspension with 9 ml of ice cold 0.3 M sucrose. The sample was centrifuged at 26,000 g for 20 minutes in the Sorvall RC2-B. The supernatant was decanted and the pellet resuspended in 3 ml of ice-cold 0.3 M sucrose. The washing procedure was repeated once. The pellet was resuspended in 2 ml of ice cold glass-distilled water and vortexed after the second wash. The lysed vesicles were centrifuged at 26,000 g for 20 minutes. Five tenths ml of the supernatant was added to 15 ml of Bray's medium for the determination of radioactivity.

Tissue catecholamines were extracted by adding 1 ml of the crude homogenate to 1 ml of 0.8 N perchloric acid and centrifuged at 40,000 g for 20 minutes. The supernatant was used for the fluorometric determination of catecholamine content. Extravesicular catecholamines were extracted by adding the supernatant from the 26,000 g centrifuged crude homogenate to an equal volume of 0.8 N perchloric acid. The precipitated protein was removed by centrifuging at 40,000 g for 20 minutes and the supernatant was used for the fluorometric determination.

The units of uptake were expressed as μg/1000 μg of total catecholamines.
Amine Efflux from Adrenal Vesicles

The method was modified from the procedure of Slotkin et al. (1971). Two steps were involved in this experiment. The first step involved the uptake of radioactively labeled amines by the vesicles. The methods for this has already been described in the previous section. Sixteen μg of epinephrine and metaraminol containing 1 μCi of the radioactive label were used. The experiment on efflux started immediately after the second wash of the vesicles with 0.3 M sucrose in the uptake procedure. The pellet was resuspended in 5 ml of buffer containing: 0.3 M sucrose, 0.05 M Hepes, 0.05 mM iproniazid and 50 mM ATP-Mg (pH 7.4). Each suspension was divided into 1 ml aliquots for the epinephrine efflux and 2 ml aliquots for the metaraminol efflux. The samples were incubated at 37°C with constant shaking. One sample of each amine was kept on ice for 60 minutes as controls. At the end of 5, 10, 20, 30, 40 and 60 minutes, duplicate samples were removed from the water bath and efflux was stopped by the addition of 2 volumes of ice cold 0.3 M sucrose. The samples were centrifuged at 26,000 g for 10 minutes and the pellets lysed in 1 ml of glass-distilled water. They were centrifuged at 26,000 g for 20 minutes and 0.5 ml of the supernatant from each sample was added to 15 ml of Bray's medium for the determination of radioactivity.
Chapter III

RESULTS

Growth of the Adrenal Gland:

General Aspects

Relationship Between Adrenal and Body Weight

The relationship between the growth of the adrenal glands and the overall growth of piglets was investigated by plotting the log of adrenal weights as a function of the log of body weights as shown in Figure 9. Data from a total of 295 piglets were used ranging from 0.5 kg to 11.0 kg in body weights. The curve indicates a linear relationship between the two parameters with a coefficient of determination ($r^2$) equal to 0.8372. The equation of the line was found to be: $\log_{10} y = 2.30 + 0.628 (\log_{10} x)$.

Growth of the Medulla in Relationship to the Cortex

Table III presents a comparison of medullary volume as a percentage of the cortical volume in relationship to piglet age. The data indicate no marked change in percentage volume of the medulla between piglets of different ages. The percentage medullary volume of 0-day-old piglets reported in the table is an average of two observations, ranging from 26 to 37 percent. Hence the 10%
Figure 9. Relationship between body and adrenal weight.
### TABLE III

Percent of Medullary Volume in Terms of Cortical Volume as Related to Species and Age

<table>
<thead>
<tr>
<th>Species</th>
<th>N</th>
<th>Age</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Swine</td>
<td>2</td>
<td>0 days</td>
<td>32</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>3 days</td>
<td>33</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>7 days</td>
<td>37</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>14 days</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>70 days</td>
<td>24</td>
</tr>
<tr>
<td>Sheep</td>
<td>1</td>
<td>Adult</td>
<td>24(^{a})</td>
</tr>
<tr>
<td>Cat</td>
<td></td>
<td>Adult</td>
<td>5(^{b})</td>
</tr>
<tr>
<td>Dog</td>
<td></td>
<td>Adult</td>
<td>20(^{b})</td>
</tr>
<tr>
<td>Rat</td>
<td></td>
<td>Adult</td>
<td>5(^{b})</td>
</tr>
<tr>
<td>Rabbit</td>
<td></td>
<td>Adult</td>
<td>2.5(^{b})</td>
</tr>
<tr>
<td>Guinea pig</td>
<td></td>
<td>Adult</td>
<td>1.5(^{b})</td>
</tr>
</tbody>
</table>

\(^{a}\) This statistic was obtained in this laboratory; procedures were similar to that used for the swine.

\(^{b}\) Modified from West, 1955.
difference between the newborn and 14-day-old piglet may not be significant as the percentage volumes are quite variable between piglets of the same age. The same table also shows interspecies comparisons of percent medullary volumes. The data on the dog, cat, rat, rabbit and guinea pig are acquired from West (1955). The swine, sheep and dog have similar size adrenal medullae and have the highest percentages. The cat and rat rank second, with percent medullary volumes about 1/4 of those in swine. The rabbit follows and the guinea pig has the smallest percent medullary volume.

Relationship Between Catecholamine Content and Piglet Age

The catecholamine content and percentage of epinephrine in adrenal glands of neonatal and juvenile swine are presented in Table IV. More than one litter of pigs were used among all the ages studied. This took into account the variability existing between litters. The patterns of ontogenesis of the catecholamines are illustrated in the following figures where the ordinate of each is expressed as a percentage of the mean values of the 150-day-old pigs.

Figure 10 shows the pattern of norepinephrine changes with age. The norepinephrine content was about 1.7 times higher at birth than at 150 days of age. There was an increase in content from birth to a peak at 14 days of age. A gradual decline followed and the content
### TABLE IV

Mean Body Weights and Adrenal Gland Catecholamine (CA) Content\(^a\) Correlated with Piglet Age

<table>
<thead>
<tr>
<th>Piglet Age</th>
<th>Litters, N</th>
<th>Piglets, N</th>
<th>Body wt., kg ± 1 S.E.M.</th>
<th>Total CA, mg/g ± 1 S.E.M.</th>
<th>Epinephrine % ± 1 S.E.M.</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 day</td>
<td>4</td>
<td>9</td>
<td>1.12 ± 0.12</td>
<td>2.29 ± 0.14</td>
<td>28 ± 1</td>
</tr>
<tr>
<td>3 days</td>
<td>4</td>
<td>9</td>
<td>1.63 ± 0.13</td>
<td>2.57 ± 0.09</td>
<td>30 ± 2</td>
</tr>
<tr>
<td>7 days</td>
<td>4</td>
<td>9</td>
<td>2.14 ± 0.14</td>
<td>2.73 ± 0.15</td>
<td>36 ± 2</td>
</tr>
<tr>
<td>14 days</td>
<td>4</td>
<td>9</td>
<td>3.48 ± 0.18</td>
<td>3.69 ± 0.17</td>
<td>36 ± 1</td>
</tr>
<tr>
<td>39 days</td>
<td>6</td>
<td>11</td>
<td>8.49 ± 0.82</td>
<td>4.16 ± 0.29</td>
<td>48 ± 1</td>
</tr>
<tr>
<td>70 days</td>
<td>3</td>
<td>5</td>
<td>38.60 ± 0.50</td>
<td>2.92 ± 0.40</td>
<td>43 ± 2</td>
</tr>
<tr>
<td>150 days</td>
<td>3</td>
<td>5</td>
<td>78.60 ± 4.00</td>
<td>2.15 ± 0.28</td>
<td>56 ± 3</td>
</tr>
</tbody>
</table>

\(^a\)Catecholamine content is expressed as per unit of whole gland (cortex + medulla).
Figure 10. The change of norepinephrine content as a function of age.
at 70 days of age was approximately equal to that at birth.

Figure 11 shows the changes in pattern of epinephrine content with age. The epinephrine content at birth was approximately 50% of that at 150 days of age. An increase in content from birth to a peak level at 39 days of age was followed by a gradual decline. The epinephrine content at 70 days of age was two times higher than that at birth.

Figure 12 shows the pattern of total catecholamine changes with age. This figure represents the composite changes of norepinephrine and epinephrine. The total catecholamine content at birth was approximately equal to that at 150 days of age. There was a marked increase in content between birth and 14 days of age. This was followed by a more gradual increase which reached a peak at 39 days of age doubling the content at birth. The content at 70 days of age decreased to a slightly higher level than that at birth.

Figure 13 shows the pattern of percentage changes in epinephrine with age. The percentage epinephrine content at 150 days of age was twice that at birth. An increase in content from birth to 7 days of age was followed by a more gradual rise to reach a plateau at 39 days of age. The content at 70 days of age was slightly lower than that at 39 days of age.
Figure 11. The change of epinephrine content as a function of age.
Figure 12. The change of catecholamine content as a function of age.
Figure 13. The change of epinephrine content as a function of age.
Ontogenic Patterns of Catecholamine Biosynthetic Enzymes

Tyrosine Hydroxylase (TH) Activity and Piglet Age

Table V presents the mean specific activity of TH correlated with piglet age. The ontogenic pattern of the activity of this enzyme is illustrated in Figure 14. The activity of TH was lower at birth than that at 150 days of age. There was an increase in activity from birth to reach a peak at 3 days of age. This was followed by a rapid decline between 3 and 14 days of age and a more gradual decline between 14 and 70 days of age. The activity at 70 days of age was approximately 50% of that at 150 days of age.

DOPA Decarboxylase (DC) Activity and Piglet Age

Table VI presents the mean specific activity of DDC correlated with piglet age. The ontogenic pattern of the activity of this enzyme is illustrated in Figure 15. The activity of DDC was slightly lower at birth than that at 150 days of age. There was a gradual increase in activity from birth to 70 days of age. The activity at 70 days of age was about 1.5 times higher than that at 150 days of age.
### TABLE V

Mean Specific Activity of Tyrosine Hydroxylase in Adrenal Homogenates Correlated with Piglet Age.

<table>
<thead>
<tr>
<th>Piglet Age</th>
<th>Litters, N</th>
<th>Piglets, N</th>
<th>Nanomoles product/g/hr ± 1 S.E.M.</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 day</td>
<td>4</td>
<td>17</td>
<td>2362 ± 184</td>
</tr>
<tr>
<td>3 days</td>
<td>4</td>
<td>17</td>
<td>2928 ± 264</td>
</tr>
<tr>
<td>7 days</td>
<td>4</td>
<td>18</td>
<td>2674 ± 186</td>
</tr>
<tr>
<td>14 days</td>
<td>4</td>
<td>19</td>
<td>2224 ± 169</td>
</tr>
<tr>
<td>39 days</td>
<td>6</td>
<td>6</td>
<td>2166 ± 206</td>
</tr>
<tr>
<td>70 days</td>
<td>3</td>
<td>5</td>
<td>1510 ± 266</td>
</tr>
<tr>
<td>150 days</td>
<td>3</td>
<td>5</td>
<td>2827 ± 233</td>
</tr>
</tbody>
</table>

### TABLE VI

Mean Specific Activity of DOPA Decarboxylase in Adrenal Homogenates Correlated with Piglet Age

<table>
<thead>
<tr>
<th>Piglet Age</th>
<th>Litters, N</th>
<th>Piglets, N</th>
<th>Nanomoles product/g/hr ± 1 S.E.M.</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 day</td>
<td>4</td>
<td>8</td>
<td>6860 ± 422</td>
</tr>
<tr>
<td>3 days</td>
<td>4</td>
<td>8</td>
<td>8358 ± 624</td>
</tr>
<tr>
<td>7 days</td>
<td>4</td>
<td>8</td>
<td>7403 ± 457</td>
</tr>
<tr>
<td>14 days</td>
<td>4</td>
<td>8</td>
<td>8062 ± 477</td>
</tr>
<tr>
<td>39 days</td>
<td>6</td>
<td>6</td>
<td>8983 ± 934</td>
</tr>
<tr>
<td>70 days</td>
<td>3</td>
<td>8</td>
<td>11643 ± 393</td>
</tr>
<tr>
<td>150 days</td>
<td>3</td>
<td>5</td>
<td>8351 ± 936</td>
</tr>
</tbody>
</table>
Figure 14. The change of specific activity of tyrosine hydroxylase as a function of age.
Figure 15. The change of specific activity of DOPA decarboxylase as a function of age.
Dopamine-ß-Hydroxylase (DBH) Activity and Piglet Age

Table VII presents the mean specific activity of DBH correlated with piglet age. The ontogenic pattern of the activity of this enzyme is illustrated in Figure 16. The activity of DBH at birth was about 60% of that at 150 days of age. There was a marked increase in activity between birth and 3 days of age. The increase reached a plateau at about 14 days of age and remained the same to 150 days of age.

Phenethanolamine-N-Methyltransferase (PNMT) Activity and Piglet Age

Table VIII presents the mean specific activity of PNMT correlated with piglet age. The ontogenic pattern of the activity of this enzyme is illustrated in Figure 17. The activity of PNMT at birth was about 60% of that at 150 days of age. There was a marked increase in activity between birth and 3 days of age. This was followed by a more gradual rise reaching a peak at 39 days of age. The activity of the enzyme had decreased to the 3-day-old level by 70 days of age.

Apparent Kinetic Patterns of the Catecholamine Biosynthetic Enzymes and Piglet Age

Michaelis-Menten kinetic parameters of the catecholamines biosynthetic enzymes were measured on adrenal homogenates in order to further characterize their ontogenic changes in activity. Table IX summarizes the results
### TABLE VII

Mean Specific Activity of Dopamine-β-Hydroxylase in Adrenal Homogenate Correlated with Piglet Age

<table>
<thead>
<tr>
<th>Piglet Age</th>
<th>Litters, N</th>
<th>Piglets, N</th>
<th>Nanomoles product/g/hr ± 1 S.E.M.</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 day</td>
<td>4</td>
<td>8</td>
<td>44200 ± 3400</td>
</tr>
<tr>
<td>3 days</td>
<td>4</td>
<td>8</td>
<td>62000 ± 2500</td>
</tr>
<tr>
<td>7 days</td>
<td>4</td>
<td>8</td>
<td>65500 ± 1200</td>
</tr>
<tr>
<td>14 days</td>
<td>4</td>
<td>8</td>
<td>67900 ± 6400</td>
</tr>
<tr>
<td>39 days</td>
<td>6</td>
<td>6</td>
<td>73500 ± 4400</td>
</tr>
<tr>
<td>70 days</td>
<td>3</td>
<td>8</td>
<td>67800 ± 1900</td>
</tr>
<tr>
<td>150 days</td>
<td>3</td>
<td>5</td>
<td>72000 ± 2200</td>
</tr>
</tbody>
</table>

### TABLE VIII

Mean Specific Activity of Phenelthanolamine-N-Methyltransferase in Adrenal Homogenate Correlated with Piglet Age

<table>
<thead>
<tr>
<th>Piglet Age</th>
<th>Litters, N</th>
<th>Piglets, N</th>
<th>Nanomoles product/g/hr ± 1 S.E.M.</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 day</td>
<td>4</td>
<td>8</td>
<td>316 ± 16</td>
</tr>
<tr>
<td>3 days</td>
<td>4</td>
<td>8</td>
<td>461 ± 41</td>
</tr>
<tr>
<td>7 days</td>
<td>4</td>
<td>9</td>
<td>472 ± 24</td>
</tr>
<tr>
<td>14 days</td>
<td>4</td>
<td>10</td>
<td>496 ± 24</td>
</tr>
<tr>
<td>39 days</td>
<td>6</td>
<td>6</td>
<td>625 ± 42</td>
</tr>
<tr>
<td>70 days</td>
<td>3</td>
<td>5</td>
<td>440 ± 33</td>
</tr>
<tr>
<td>150 days</td>
<td>3</td>
<td>4</td>
<td>528 ± 38</td>
</tr>
</tbody>
</table>
Figure 16. The change of specific activity of DOPA-ß-hydroxylase as a function of age.
Figure 17. The change of specific activity of phenethanolamine-N-methyltransferase as a function of age.
### TABLE IX

**Apparent Michaelis-Menten Kinetic Parameters of Catecholamine Biosynthetic Enzymes in Adrenal Homogenate Correlated with Piglet Age**

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Age, Days</th>
<th>Vmax ± 1 S.E.M. (^a)</th>
<th>Km ± 1 S.E.M.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tyrosine hydroxylase</td>
<td>0</td>
<td>84.3 ± 6.7</td>
<td>2.23 ± 0.35 x 10(^{-4}) M</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>65.2 ± 1.4</td>
<td>1.37 ± 0.08 x 10(^{-4}) M</td>
</tr>
<tr>
<td></td>
<td>70</td>
<td>40.9 ± 0.8</td>
<td>1.31 ± 0.06 x 10(^{-4}) M</td>
</tr>
<tr>
<td>DOPA decarboxylase</td>
<td>0</td>
<td>244.0 ± 18.8</td>
<td>1.94 ± 0.50 x 10(^{-4}) M</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>248.7 ± 12.7</td>
<td>3.50 ± 0.41 x 10(^{-4}) M</td>
</tr>
<tr>
<td></td>
<td>70</td>
<td>389.1 ± 33.8</td>
<td>5.31 ± 0.94 x 10(^{-4}) M</td>
</tr>
<tr>
<td>Dopamine-β-hydroxylase</td>
<td>0</td>
<td>274.4 ± 16.2</td>
<td>1.68 ± 0.24 x 10(^{-3}) M</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>314.4 ± 2.2</td>
<td>1.38 ± 0.03 x 10(^{-3}) M</td>
</tr>
<tr>
<td></td>
<td>70</td>
<td>349.7 ± 4.4</td>
<td>1.28 ± 0.05 x 10(^{-3}) M</td>
</tr>
<tr>
<td>Phenylethanolamine-N-methyltransferase</td>
<td>0</td>
<td>12.6 ± 0.5</td>
<td>1.68 ± 0.20 x 10(^{-4}) M</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>17.2 ± 0.8</td>
<td>1.29 ± 0.16 x 10(^{-4}) M</td>
</tr>
<tr>
<td></td>
<td>70</td>
<td>14.0 ± 0.7</td>
<td>1.60 ± 0.23 x 10(^{-4}) M</td>
</tr>
</tbody>
</table>

\(^a\)Both Km and Vmax are estimated by the method of non-linear least squares. Each mean represents a pooled average of 16 piglets from 4 litters for the 0-day-olds, 8 piglets from 4 litters for the 14-day-olds, and 4 piglets from 4 litters for the 70-day-olds.

\(^b\)Units of Vmax are in nanomoles product/mg protein/hr.
obtained from these studies.

The Vmax of TH decreased with age; the Vmax of TH in the 70-day-old pig was about 50% that of the 0-day-old piglet. The apparent Km of TH decreased about 35% between birth and 14 days of age but stayed at the same level between 14 and 70 days of age.

There was no change in the Vmax of DDC between 0 and 14 days of age, but the Vmax was increased at 70 days of age. The apparent Km of DDC increased with age; the apparent Km of the 70-day-old pigs was about 3 times higher than that of the 0-day-old piglets.

The Vmax of DBH increased gradually between birth and 70 days of age. The apparent Km of DBH stayed constant throughout the three ages.

There was a slight increase in the Vmax of PNMT between birth and 14 days of age. The Vmax decreased to approximately the level of that at birth at 70 days of age. The apparent Km of PNMT stayed at approximately the same level among the three ages.

Synthesis of Catecholamines by

Adrenal Slices in vitro

Effect of α-Methyl-p-Tyrosine (αMpt)
on the in vitro Biosynthesis of
Catecholamines

Figure 18 illustrates the in vitro effect of αMpt on the activity of TH in adrenal homogenate from a 14-day-old
Figure 18. In vitro inhibition of tyrosine hydroxylase by α-methyl-p-tyrosine. (The inhibition is expressed as a percent of the activity of the enzyme without α-methyl-p-tyrosine.)
piglet. The drug inhibited 20% of the enzyme activity at $5 \times 10^{-7}$ M and 100% at $1 \times 10^{-3}$ M. To determine the effect of αMPT on catecholamine biosynthesis, adrenal slices from a 14-day-old piglet were incubated with the drug. Figure 19 illustrates the results of this study. The catecholamines isolated in the 2% boric acid fraction were expressed as a percentage of the control value. The inhibitor, αMPT, caused an 80% inhibition of the biosynthesis of catecholamines at $1 \times 10^{-6}$ M. The inhibition was complete at $1 \times 10^{-3}$ M.

Adrenal and Plasma Tyrosine Concentrations and Piglet Age

Adrenal and plasma tyrosine concentrations were measured with age to determine the amount of tyrosine to be used in the in vitro catecholamine biosynthesis study. The results are presented in Table X. There were substantial differences of tyrosine concentrations between ages in both the adrenals and the plasma. Adrenal tyrosine concentration decreased with age. The tyrosine level at birth was 3.5 times higher than that at 70 days of age. Plasma tyrosine concentration followed a similar pattern, but with more marked differences. The tyrosine level of the newborns was 6 times higher than that of 70-day-old pigs.

Ontogenesis of in vitro Catecholamine Biosynthesis

Tyrosine concentrations determined from plasma were
Figure 19. In vitro inhibition of catecholamine biosynthesis by α-methyl-p-tyrosine. (The inhibition is expressed as a percent of the amount of catecholamine synthesized without α-methyl-p-tyrosine.)
TABLE X

Mean Tyrosine Concentration in Plasma and Adrenal Glands Correlated with Age

<table>
<thead>
<tr>
<th>Age Days</th>
<th>Litters, N</th>
<th>Piglets, N</th>
<th>Adrenal Tyrosine, mg/100 ml ± 1 S.E.M.</th>
<th>Piglets, N</th>
<th>Plasma Tyrosine, mg/100 ml ± 1 S.E.M.</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>2</td>
<td>6</td>
<td>5.35 ± 0.66</td>
<td>6</td>
<td>7.12 ± 0.79</td>
</tr>
<tr>
<td>14</td>
<td>2</td>
<td>6</td>
<td>3.73 ± 0.23</td>
<td>6</td>
<td>2.89 ± 0.15</td>
</tr>
<tr>
<td>70</td>
<td>2</td>
<td>4</td>
<td>1.54 ± 0.06</td>
<td>3</td>
<td>1.20 ± 0.04</td>
</tr>
</tbody>
</table>
used to incubate with the adrenal slices. The results are summarized in Table XI. There was no significant difference between the amount of catecholamines synthesized by adrenal slices from 0- or 14-day-old piglets. However, the amount of catecholamines synthesized by the slices from these two ages were significantly higher than that synthesized by the slices from 70-day-old pigs. Adrenal slices of the 0- and 14-day-old piglets synthesized 5 times more catecholamines than the 70-day-old pigs at a tyrosine concentration of 7.12 mg/100 ml. Increasing the concentration of tyrosine in the incubation medium tended to increase the amount of catecholamines synthesized by the adrenal slices of the 14- and 70-day-old pigs.

Ontogenesis of in vitro Amine Uptake and Efflux by Isolated Adrenal Vesicles

Relationship of Amine Uptake with Piglet Age

Table XII summarizes the results of the uptake of epinephrine with and without reserpine by adrenal vesicles of 0-, 14- and 70-day-old pigs. The results of the uptake of metaraminol with and without reserpine by adrenal vesicles of similar aged pigs are presented in Table XIII. A composite graph of the uptake of epinephrine and metaraminol with and without reserpine by the vesicles of the 0-day-old piglets is shown in Figure 20. The uptake of both amines increased in a linear fashion with increasing
# TABLE XI

Mean Catecholamine Synthesis Correlated with Tyrosine Concentration and Piglet Age

<table>
<thead>
<tr>
<th>Age Days</th>
<th>Litters, N</th>
<th>Tyrosine Conc., mg/100 ml</th>
<th>Piglets, N</th>
<th>Nanomoles of Catecholamines/g tissue/hr ± 1 S.E.M.</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>2</td>
<td>7.12</td>
<td>4</td>
<td>1437.25 ± 156.38</td>
</tr>
<tr>
<td>14</td>
<td>2</td>
<td>2.80</td>
<td>4</td>
<td>1358.00 ± 111.69</td>
</tr>
<tr>
<td>70</td>
<td>2</td>
<td>1.20</td>
<td>4</td>
<td>135.20 ± 11.67</td>
</tr>
<tr>
<td>70</td>
<td>2</td>
<td>2.89</td>
<td>2</td>
<td>160.00 ± 1.00</td>
</tr>
<tr>
<td>70</td>
<td>2</td>
<td>7.12</td>
<td>2</td>
<td>265.00 ± 39.00</td>
</tr>
</tbody>
</table>

*The concentrations are expressed in mg/1000 ml of adrenal extracts.*
TABLE XII

Effect of Piglet Age on Vesicular Uptake of Epinephrine (10 min. incubation) in the Presence or Absence of Reserpine

<table>
<thead>
<tr>
<th>Epinephrine</th>
<th>Reserpine Presence/Absence</th>
<th>Litters, N</th>
<th>0 Day</th>
<th>14 Days</th>
<th>70 Days</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 g</td>
<td>0</td>
<td>2</td>
<td>529.3 ± 29.8</td>
<td>788.1 ± 87.7</td>
<td>872.6 ± 65.5</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>2</td>
<td>24.4 ± 4.6</td>
<td>11.9 ± 2.6</td>
<td>11.7 ± 2.5</td>
</tr>
<tr>
<td>2 g</td>
<td>0</td>
<td>2</td>
<td>1014.2 ± 18.2</td>
<td>1474.1 ± 151.9</td>
<td>1754.4 ± 125.6</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>2</td>
<td>25.7 ± 6.6</td>
<td>32.0 ± 6.0</td>
<td>26.1 ± 2.4</td>
</tr>
<tr>
<td>4 g</td>
<td>0</td>
<td>2</td>
<td>2304.3 ± 69.0</td>
<td>2469.6 ± 242.3</td>
<td>3332.0 ± 230.6</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>2</td>
<td>78.9 ± 6.1</td>
<td>47.6 ± 15.2</td>
<td>55.1 ± 5.6</td>
</tr>
<tr>
<td>8 g</td>
<td>0</td>
<td>2</td>
<td>3904.8 ± 247.1</td>
<td>3790.3 ± 350.1</td>
<td>5841.5 ± 1018.4</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>2</td>
<td>157.6 ± 49.3</td>
<td>126.0 ± 42.4</td>
<td>111.5 ± 24.1</td>
</tr>
</tbody>
</table>

Data for 0 day piglets represent 3 determinations each of which is a pool of tissues from 4 piglets. Units of uptake are expressed in nanograms/1000 µg of endogenous catecholamines.
<table>
<thead>
<tr>
<th>Metaraminol (µg)</th>
<th>Reserpine Presence/Absence</th>
<th>Litters, N</th>
<th>0 Day</th>
<th>14 Days</th>
<th>70 Days</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 µg</td>
<td>O</td>
<td>2</td>
<td>161.2 ± 19.7</td>
<td>301.5 ± 41.5</td>
<td>256.5 ± 4.3</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>2</td>
<td>111.7 ± 7.6</td>
<td>102.1 ± 24.6</td>
<td>127.8 ± 19.5</td>
</tr>
<tr>
<td>2 µg</td>
<td>O</td>
<td>2</td>
<td>340.8 ± 16.9</td>
<td>660.7 ± 73.3</td>
<td>427.2 ± 8.7</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>2</td>
<td>254.7 ± 13.4</td>
<td>205.0 ± 43.3</td>
<td>292.4 ± 14.9</td>
</tr>
<tr>
<td>4 µg</td>
<td>O</td>
<td>2</td>
<td>650.2 ± 43.3</td>
<td>1160.2 ± 59.1</td>
<td>1033.2 ± 83.0</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>2</td>
<td>503.8 ± 12.0</td>
<td>414.5 ± 96.9</td>
<td>541.3 ± 13.3</td>
</tr>
<tr>
<td>8 µg</td>
<td>O</td>
<td>2</td>
<td>1331.6 ± 143.5</td>
<td>2264.3 ± 202.1</td>
<td>1882.3 ± 169.7</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>2</td>
<td>1109.0 ± 76.8</td>
<td>877.0 ± 225.1</td>
<td>1132.1 ± 115.9</td>
</tr>
</tbody>
</table>

Data for 0 day piglets represent 3 determinations each of which is a pool of tissues from 4 piglets. Units of uptake are expressed in nanograms/1000 µg of endogenous catecholamines.
Figure 20. Amine uptake by adrenal vesicles of 0-day-old swine with and without reserpine.

Units of uptake are expressed as nanograms/1000\mu g of endogenous catecholamines.
concentrations of the amines. All of the curves pass through 0. The uptake of metaraminol at 8 μg/ml was 34% of that of epinephrine. Reserpine inhibited 95% of the uptake of epinephrine but only 30% of that of metaraminol. Since the uptake of the amines as a function of concentration has been shown to be linear, the slopes should reflect the rate of uptake of amines by the vesicles. An ontogenic comparison of the rate of uptake of epinephrine and metaraminol are presented in Table XIV. There was no significant difference in the uptake of epinephrine between 0- and 14-day-old piglets. However, the uptake of epinephrine by the 70-day-old pigs was significantly higher than the uptake by the 0- and 14-day-old piglets. There was no difference between the 14- and 70-day-old pigs in the uptake of metaraminol, whereas the 0-day-old pigs exhibited a lower rate of metaraminol uptake. Reserpine exerted a more profound effect on the uptake of metaraminol by 14- and 70-day-old than by the 0-day-old pigs.

Relationship of Amine Efflux with Age

The storage of amines in vesicles involves the uptake minus the efflux of the amines. Therefore, the efflux component was studied in order to characterize the storage process. The results were expressed as the log of the percentage of amines remaining in the vesicles as a function
TABLE XIV
Slopes of Uptake Curves Correlated with Age

<table>
<thead>
<tr>
<th>Amine</th>
<th>Slope$^a$ ± 1 S.E.M.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 Day</td>
</tr>
<tr>
<td>Epinephrine</td>
<td>0.485 ± 0.027</td>
</tr>
<tr>
<td>Epinephrine + Reserpine</td>
<td>0.020 ± 0.004</td>
</tr>
<tr>
<td>Metaraminol</td>
<td>0.166 ± 0.013</td>
</tr>
<tr>
<td>Metaraminol + Reserpine</td>
<td>0.142 ± 0.007</td>
</tr>
</tbody>
</table>

$^a$The slopes are calculated by the least squares method; the unit of each slope is expressed as 1,000 μg total catecholamines$^{-1}$. 
of time. They were analyzed by linear regression and presented in Table XV. There was no difference in the efflux of epinephrine by the vesicles between the ages. The efflux of metaraminol by the vesicles were also similar between the ages. Hence, the uptake of einedhrine and metaraminol is a good indication of the storage capacity of the amines in the pig.
<table>
<thead>
<tr>
<th>Age, Days</th>
<th>Amine</th>
<th>Amine Conc. at time 0&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Slope ± S.E.M.&lt;sup&gt;b&lt;/sup&gt;</th>
<th>r&lt;sup&gt;2&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Epinephrine</td>
<td>2942.6</td>
<td>-0.0107 ± 0.00216</td>
<td>0.86</td>
</tr>
<tr>
<td></td>
<td>Metaraminol</td>
<td>948.1</td>
<td>-0.0156 ± 0.00390</td>
<td>0.76</td>
</tr>
<tr>
<td>14</td>
<td>Epinephrine</td>
<td>5680.6</td>
<td>-0.0125 ± 0.00207</td>
<td>0.88</td>
</tr>
<tr>
<td></td>
<td>Metaraminol</td>
<td>1182.0</td>
<td>-0.0161 ± 0.00431</td>
<td>0.74</td>
</tr>
<tr>
<td>70</td>
<td>Epinephrine</td>
<td>6855.6</td>
<td>-0.0124 ± 0.00282</td>
<td>0.80</td>
</tr>
<tr>
<td></td>
<td>Metaraminol</td>
<td>2251.0</td>
<td>-0.0128 ± 0.00348</td>
<td>0.73</td>
</tr>
</tbody>
</table>

<sup>a</sup>Each figure represents a single determination on a pooled sample from 4 piglets (0 days, 14 days) or 2 piglets (70 days). Concentrations are expressed as nanograms/1000 g of endogenous catecholamines.

<sup>b</sup>The units of the slope are log % remaining/unit of time.
Chapter IV

DISCUSSION

Three ages of pigs were chosen for comparison in this study. They were 0, 14 and 70 days of age, representing respectively the birth, preweaning and postweaning stages of development of the pig. Approximately 25% of the postnatal deaths occur within one day of birth and over 75% occur within one to two weeks after birth. There is little change in viability between weaning at 21-56 days and market at 154 days of age (Stanton and Carroll, 1974). These three ages, therefore, represent important phases in the survival of the piglet.

Most of the parameters for comparison in the studies were based on content per gram (wet weight) of the whole gland. Perhaps it might have been better to make comparisons on a total-content-per-gland basis because the water content of the glands may vary with age. However, this could not be done because whole gland weights were not always recorded. Since the percentage medullary volume in terms of the cortex was about the same in all the ages studied and the gland weight increased linearly with body weight, the comparisons based on content per gram (wet weight) should reflect changes with age, although these
comparisons may not be as apparent as whole gland levels.

The percentage volume of the adrenal medulla in terms of the cortex varies widely from species to species (Table III). Therefore, interspecies comparison of hormone and enzyme levels per unit of whole glands may be misleading unless corrections for percent medullary volumes are made. For example, interspecies comparison of catecholamine levels based on content per gram of whole glands has indicated that the swine and the sheep have higher levels than that of the cat and the dog. The latter two species, in turn, have higher levels than that of the rat, rabbit and guinea pig. However, after adjusting the levels of percent medullary volume, catecholamine contents are similar in all the species.

The patterns of ontogenic changes in catecholamine content of swine adrenal glands indicate that they reach a maximum during late neonatal or juvenile periods and gradually decline to lower stable levels in the adult (Figures 10-12). Similar patterns have also been found in the rabbit, guinea pig, calf and fowl (Shepherd and West, 1951; Comline and Silver, 1966b; Comline and Silver, 1971). A different pattern has been reported in cats and dogs where catecholamine levels of these two species continued to rise from birth to adulthood (Shepherd and West, 1951). The catecholamine levels in rat adrenal glands increase steadily from 4 to 15 weeks of age (Patrick and Kirshner, 1972). All of the species discussed resemble
each other to the extent that the epinephrine:norepinephrine ratio increases during development.

Tyrosine hydroxylase has been established as the rate-limiting step of norepinephrine biosynthesis in the guinea pig heart (Levitt et al., 1965). There is evidence in this thesis that TH may be the rate-limiting enzyme in the swine adrenal gland as well, although the definitive experiments comparing apparent Km and Vmax in purified preparations have not yet been done. The apparent Km of the enzymes presented in Table IX shows that TH needs a lesser amount of substrate than DBH and DDC to reach half-maximal velocity. In addition, TH has the lowest Vmax compared to DDC and DBH. αMpt, which abolished the activity of TH at 1 x 10^{-3}M concentration (Figure 18), also inhibited 98% of the synthesis of catecholamines (Figure 19). However, it is interesting to note that αMpt inhibited 82% of the biosynthesis of catecholamines while only blocking 25% of the specific activity of TH at the same concentration. Therefore, the inhibitory action of αMpt on catecholamine synthesis may not be confined to TH alone. PNMT has approximately the same apparent Km as TH but has a markedly lower Vmax. Hence, PNMT appears to be the rate-limiting enzyme of epinephrine biosynthesis in the swine adrenal medulla. This postulation is also supported by the parallel pattern of ontogenesis of PNMT and epinephrine (Figures 11 and 17). Since αMpt inhibited catecholamine biosynthesis by 82% and only 25%
of TH activity as discussed above, it is possible that MeT has an inhibitory effect on PNMT, as epinephrine is one of the major catecholamines in the swine adrenal gland.

The specific activity of TH in the pig declined from birth through 70 days of age and returned at 150 days of age to the level observed at term (Figure 14). The ontogenic changes of the activity of this enzyme in the rat follows a different pattern. TH activity increases steadily with age between 4 and 15 weeks, which parallels the increase of catecholamines (Patrick and Kirshner, 1972). No differences have been observed in TH activity between fetal and adult sheep (Friedman, 1972). However, only two ages of sheep were studied by this investigator. It is difficult, therefore, to establish a developmental pattern for this species. The apparent Km of TH in the newborn piglet was found to be higher than that of the 14- and 70-day-old pigs (Table IX). Possible explanations include the presence of higher amounts of inhibitors in the newborn adrenals, the presence of activators in the adrenals of the older pigs, and the different rates of development of possible isozymes. Since the apparent Km was estimated from crude preparations, these results represent trends only and may not mimic the pattern seen with more purified preparations.

The specific activity of DDC in the pig increased gradually with age from birth through 70 days of age and returned to neonatal levels at 150 days of age (Figure 15).
The apparent Km of this enzyme also increased with age (Table IX). DDC has a higher apparent KM and Vmax compared to TH and PNMT. Hence this enzyme apparently has no obvious regulatory role on catecholamine biosynthesis in the swine adrenal gland.

The specific activity of DBH increased markedly from birth to a maximum during late neonatal age and remained stable through 150 days of age in the pig (Figure 16). The ontogenic pattern of this enzyme in the rat, however, is quite different. There is a gradual increase in activity between 4 and 15 weeks of age which parallels the increase in catecholamines (Patrick and Kirshner, 1972). A similar pattern has been observed between 1- and 50-day-old rats by Slotkin (1973). Although the data in Table VII represents the total activity of the enzyme, they may also reflect the population of catecholamine storage vesicles per gram of adrenal tissue at each age. This is because a large proportion of DBH is known to be bound to the vesicular membrane and the enzyme has been used as a marker for storage vesicles in the rat (Viveros et al., 1969).

The specific activity of PNMT in the pig increased markedly from birth in the early neonatal period. This was followed by a gradual increase to reach a peak in the late neonatal period. A decline ensued through 70 days of age but returned to neonatal levels at 150 days of age (Figure 17). Parallel ontogenic patterns between PNMT and
epinephrine levels are apparent by comparing Figures 11 and 17. The appearance of PNMT activity in the fetal rat has been correlated with the beginning of adrenocortical function (Fuller and Hunt, 1967). However, there is apparently no relationship between the development of adrenal PNMT activity observed in our piglet studies and the ontogenic changes in plasma 17-hydroxycorticosteroid-producing capacity of the swine adrenal cortex by Dvorak (1972). This investigator reported a rapid decrease in adrenocortical function after birth reaching a low level at 25 days and maintained through 75 days of age. Unfortunately, no information is available on the ontogenesis of the venous output of corticosteroids from the adrenal cortex of the swine to enable a more definitive comparison. The rat has been observed to follow a different ontogenic pattern of PNMT activity as the enzyme decreases in activity with age (Philpott et al., 1969).

The determination of tyrosine levels made possible the use of physiological levels of this amino acid in the \textit{in vitro} biosynthesis of catecholamines. Levels of tyrosine between 0, 14 and 70 days of age show marked differences in both plasma and adrenal glands of the swine (Table X). The content of tyrosine decreased with age in both. All of the levels of plasma tyrosine are nevertheless within the same range as that observed in the rat (Dairman, 1971). The difference in diet between the newborn and the 70-day-old pig undoubtedly accounts for part of the large difference in
plasma tyrosine concentration between the two ages. The baby pig received twice as much tyrosine content in its diet than the 70-day-old pig (approximated from Siers et al., 1976). The difference may also reflect the development of enzyme systems such as tyrosine transaminase, which metabolize tyrosine in the liver.

Although there is no significant difference between 0 and 14 days of age, adrenal slices from these ages were five times higher in their ability to synthesize catecholamines than those from 70 days of age (Table XI). Moreover, there appears to be a relationship between an age-dependent decrease in the synthesis of catecholamines and plasma tyrosine concentration. This may reflect a lower transport of tyrosine into the chromaffin cell, or a lower catecholamine biosynthetic ability in the older pigs. The amount of catecholamines synthesized in the 0- and 14-day-old piglets approach a plateau with higher tyrosine concentrations. This indicates that TH operates near maximal velocity in the neonatal swine adrenal gland. A similar observation has been made on the rat where the tyrosine concentration is saturating (Lovenberg and Victor, 1974).

The chromaffin storage vesicles play an important role in recapturing the catecholamines intraneuronally in the adrenal gland. Two apparently distinct systems are present within these vesicles. The one which has a high affinity for epinephrine in the adrenal medulla is identified as the slow pool whereas the one which is
relatively nonspecific and takes up metaraminol is known as the fast pool (Slotkin and Kirshner, 1971). The swine adrenal vesicles exhibit a higher rate to take up epinephrine at 70 days of age than at preweaning ages (Table XIV). Increased metaraminol uptake appeared at 14 days of age, indicating an earlier maturation of the nonspecific uptake system or the fast pool. There was no difference in the rate of efflux of amines between the ages, suggesting that the stability of binding of the amines to the storage complex remained the same (Table XV). Thus, the uptake of the amine also reflects the storage capacities of the amines in the swine. The rat adrenal vesicles exhibit an increase in the uptake of epinephrine between 20 and 30 days of age, a decrease between 30 and 40 days of age and an increase to the 30-day level at 50 days of age. The metaraminol uptake remains stable between 10 and 30 days of age but declines gradually through 50 days of age (Slotkin, 1973b). The efflux of epinephrine remains stable from 20 to 50 days of age. Therefore, the uptake of epinephrine reflects storage between 20 and 50 days of age in the rat. There are no data available on the efflux of metaraminol from rat adrenal vesicles. No change has been observed in the absolute extent of inhibition by reserpine in both uptake systems in the swine. This indicates the irreversible characteristic of the inhibition since an increased capacity for uptake in the older pigs failed to change the amount of inhibition by reserpine.
The ontogenesis of the catecholamine catabolic enzymes in swine adrenal glands have been studied in our laboratory previously. The activity of COMT increases markedly between birth and 14 days of age in the swine. This is followed by a gradual decline to the level at term and remains relatively stable between 39 and 150 days of age (Stanton et al., 1975). MAO activity in the swine increases gradually from birth to 70 days of age and remains stable through 150 days of age (Stanton et al., 1975). Recently, the development of MAO in the rat has been reported to follow a different pattern (Blatchford et al., 1976). The activity of MAO in this species is low and stable between 5 and 10 days of age, but rises markedly between 10 and 80 days of age to 5 times the activity at the 5-day-old level.

The high specific activity of TH in the adrenal homogenates and the large amount of catecholamines synthesized by the adrenal slices suggest a high biosynthetic capability of the adrenal medulla in the newborn pig. This high capability is apparently maintained throughout the neonatal period as similar data are obtained from the 14-day-old pig. However, the comparatively small rate of uptake by the adrenal vesicles of the neonate indicates a low catecholamine storage capacity in the piglet. Other supportive evidence includes the low catecholamine content and DBH activity in the adrenal glands of the neonatal piglet. Therefore, the high biosynthetic capability may be viewed
as a compensatory mechanism for the relatively low reserves of catecholamines. The amount of catecholamines synthesized by the pig is apparently also influenced by diet. Insufficient dietary tyrosine could lower the amount produced (Table XI). On the other hand, excess tyrosine in the diet of the neonate would have a less significant effect as the amount of catecholamines synthesized by the adrenal slices approaches a plateau with increasing tyrosine concentrations.

The proportion of epinephrine present in the adrenal gland is comparatively low at birth and in the early neonatal period as it is for most other species. This poses a problem for the newborn piglet which will be discussed later.

There are few ontogenic data available at present in regard to the catecholamine secretory function of the adrenal medulla in swine. Nevertheless, newborn pigs have been shown to respond to insulin-induced hypoglycemia by releasing catecholamines via reflex neuronal stimulation (Stanton et al., 1974). This action is in contrast to newborn rats which do not respond to insulin (Slotkin, 1973b).

The overall function of the adrenal medulla in the neonatal pig is thus relatively mature compared to other species. However, the coordinating ability of the adreno-sympathetic nervous system is not only dependent on the
maturity of the system, but also on the development of the effector organs and their responsiveness to activation as well. It is logical, therefore, to relate the ontogeny of the adrenosympathetic system to the development of the effector organs. Unfortunately, developmental information is lacking at present for many of these organs in the swine. Nevertheless, adequate knowledge on the ontogeny of one type of effector organ—the metabolic depots—provides useful information regarding the coordinating ability of the adrenosympathetic nervous system during stress.

Adipose tissue, a major depot for metabolic resources in times of stress, is underdeveloped in the neonatal piglet as it accounts for only 1% of the total body weight. It has been observed that the maximal lipolytic rate is low at birth and increases after 2 days of age (Mersmann et al., 1975). The adipose tissue in the neonatal piglet is not able to contribute as an energy source during stress; this is supported by the observation that plasma nonesterified fatty acids are not significantly elevated by catecholamines in piglets of this age (Stanton and Mueller, 1973a). Consequently, the newborn piglet is highly dependent on the glycogen reserves it has in the liver and skeletal muscle. However, the neonate is less responsive than older pigs to the hyperglycemic effects of catecholamines, especially norepinephrine (Stanton and Mueller, 1973b). It has been observed that norepinephrine
does not increase the metabolic rate of piglets less than 1 week of age, while epinephrine is active at birth (Stanton and Mueller, 1973a). The low percentage of epinephrine in the adrenal medulla of the neonatal piglet, therefore, may reduce the efficiency of its response to stress. Defective gluconeogenesis and poor oxidative metabolic capacity in the liver adds to the survival problem faced by the neonatal piglet.

Starvation accounts for 18% of the deaths of neonatal swine (Kernkamp, 1965). Mild fasting depletes liver glycogen in neonates of all ages, and the blood glucose level is significantly reduced in piglets less than 1 day old. It has been observed that liver glycogenolysis alone may not be adequate to balance glucose utilization during fasting (Stanton and Mueller, 1974). Another important contribution to neonatal mortality is chilling, which accounts for 5% of the deaths (Kernkamp, 1965). Cold exposure produces mobilization of glucose with a concommitant decrease in skeletal muscle glycogen but not in liver glycogen (Curtis and Rogler, 1970). It is doubtful that skeletal muscle glycogen contributes significantly to blood glucose since this tissue has insignificant glucose-6-phosphatase activity (Scrutton and Utter, 1968). Therefore, the piglet is primarily dependent on shivering and peripheral vasoconstriction to maintain body temperature (Brück, 1970; Stanton and Mueller, 1973a). These mechanisms may not be enough to keep the piglet warm since
insulation of the animal is minimal due to the lack of adipose tissue and scanty hair.

Other principal causes of death include disease (18%) due partly to the lack of antibodies in the neonatal piglet and its dependence on the colostrum of the sow. An additional problem in this respect is the low quality of maternal care the sow gives to her litter. Crushing of piglets by the sow contributes to 31% of the neonatal deaths (Kernkamp, 1965).

Thus the survival of the neonatal piglet is threatened by a number of stressors--many of which it finds difficult to withstand partly because of inherent deficiencies in metabolic reserves. The stressors may also enhance the threat of each other. For example, chilling may weaken the piglet or make it lethargic so as to impair its ability to compete for food, thereby causing the animal to starve. Starvation may predispose the animal to infection because of inadequate acquisition of antibodies from the colostrum of the sow during the first two days of life. The weakness created by these stressors may also predispose the piglet to trampling and crushing because a chilled, lethargic pig would be unable to avoid the sow as effectively as an alert animal.

The adrenosympathetic system in the pig is functional at birth and would be expected to operate as a major coordinator in the maintenance of homeostasis--a process allowing the animal to resist some of the stressors it encounters. However,
this coordinating ability may be severely hampered by the general underdevelopment and low responsiveness of the metabolic reserves to adrenergic stimulation. These observations suggest that the "dyssynchrony" in the development between the adrenosympathetic nervous system and certain of its effector organs, namely the metabolic depots, may be responsible for the low capability of neonatal piglets to withstand stress. There is very little information in regard to the development of other effector organs of the sympathetic nervous system such as the heart and the lungs. Moreover, the possibility of the sympathetic nervous system exerting excessive demands on the effector organs and depleting their vital reserves has not been examined to any extent. Therefore, investigation into these two areas may yield some valuable information on the overall coordinating ability of the adrenosympathetic nervous system in relationship with age. This may eventually lead to the establishment of a tenable theory in explaining the low capability of neonatal swine to withstand stress.
Chapter V

SUMMARY

An ontogenic characterization of the swine adrenal medulla has been conducted in this study. The purpose was to test the hypothesis that a "dyssynchrony" in the development of the adrenosympathetic nervous system with respect to its effector organs could be partly responsible for the low capability of neonatal piglets to withstand stress. A number of developmental studies on one type of effector organ--the metabolic depots--have been reported on the swine. This study is, therefore, primarily concerned with the functional ontogeny of the adrenosympathetic nervous system. The catecholamine content and specific activities of catecholamine biosynthetic enzymes in amine uptake and efflux by the adrenal medulla, as well as in vitro catecholamine biosynthesis, has been examined in pigs representing different phases of development. A cross species comparison of the functional ontogeny of the adrenal medulla has also been made in the Discussion.

Results obtained indicate that the adrenosympathetic nervous system in the pig is well developed at birth. However,
reports in the literature on the sympathetic metabolic effector organs such as the fat and glycogen depots indicate that they are relatively underdeveloped in the neonatal pig. Therefore, the coordinating action of the adrenosympathetic nervous system is severely hampered by dyssynchrony in development of this system and its effector organs. The failure to maintain homeostasis by the neonatal pig as a result of this dyssynchrony may severely impair its ability to withstand environmental stress.
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