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ADRENOCORTICAL CONTROL OF EPINEPHRINE SYNTHESIS

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TABLE OF CONTENTS

I. INTRODUCTION

Mammals are unique among vertebrates in possessing an adrenal gland organized into layers. Most chromaffin cells present in the adult mammal are confined within the adrenal medulla; this compact organ is encapsulated by another organ, the adrenal cortex, which itself contains at least three concentric strata (the zona glomerulosa, zona fasciculata, and zona reticularis). In fetal mammals and among adults of most other vertebrate classes, chromaffin cells are found in sympathetic ganglia and may also be dispersed widely throughout the body. In mammals alone most extra-adrenal chromaffin cells atrophy soon after birth (148); thereafter the adrenal medulla is the major (and possibly the only) source of circulating epinephrine (251).

The special anatomic relationship between the adrenal cortex and medulla is all the more striking in view of their disparate embryological origins and the differences in the chemical nature of their secretory products. The adrenal cortex derives from coelomic epithelium (226), and its hormones are lipid-soluble steroids; the circulating levels of the chief glucocorticoid hormone (hydrocortisone or corticosterone, depending on the species) is kept within a relatively narrow range by a closed-loop feedback system (167). The adrenal medulla derives from the neuroectoderm, and its hormone, epinephrine, is water-soluble; circulating epinephrine levels are not regulated independently but can approach zero in the unstressed animal and then rise dramatically in response to hypoglycemia or other stresses. The adrenal cortex is a true gland; its secretion is controlled by a humoral input, ACTH. The medulla is a neuro-endocrine transducer (253); it secretes its product in response to a neural input, *i.e.*, through acetylcholine released at synapses by preganglionic sympathetic neurons.

The distinctive anatomic relationship between the adrenal cortex and medulla has given rise to a host of hypotheses about possible functional interactions between the two. One such hypothesis—that adrenal glucocorticoids influence epinephrine biosynthesis in the medulla—can probably be elevated to the status of a fact; it forms the basis of this review. Studies performed during the past half-dozen years have provided compelling evidence that adrenal glucocorticoid hormones are of primary significance in the physiology of medullary chromaffin cells. The perfusion of the medulla by portal venous blood containing high concentrations of these hormones is necessary for the maintenance of a medullary enzyme, phenylethanolamine-N-methyl transferase (PNMT) (132), which catalyzes the conversion of norepinephrine to epinephrine (2). The adrenal medulla receives much of its blood supply from an intra-adrenal portal circulation (110). This blood has not yet been diluted by systemic venous blood and thus contains extremely high concentrations of hydrocortisone or corticosterone. These high hormone concentrations apparently induce the synthesis of PNMT (254, 255); they also may influence the size of medullary chromaffin cells and their contents of other specific proteins as well (148, 177, 183).

The examination of cortical-medullary interactions has probably been retarded by the tendency of life scientists to assign the study of each organ to a specific academic discipline. Thus, for many years, the adrenal cortex was the private preserve of the endocrinologist, whereas the medulla belonged within the exclusive domain of the pharmacologist. Probably the English comparative anatomists were the first group of investigators to generate viable hypotheses about the functional consequences of the juxtaposition of adrenal cortex and medulla. In 1951, Shepherd and West (220) examined the adrenals of a group of mammals and noted that in any species the fraction of total adrenal catecholamines represented by epinephrine could be correlated with the ratio of the sizes of its cortex and medulla. Soon thereafter, Coupland (48) noted that in the dogfish, a species in which the steroid-secreting cells were anatomically distant from the chromaffin cells, the latter cells lacked epinephrine and contained only norepinephrine. On the basis of these observations, both groups of investigators suggested that the

presence in mammals of a juxtaposed adrenal cortex somehow influenced the methylation of norepinephrine in the adrenal medulla (48, 220). In 1964, Lempinen (148) examined the natural history of chromaffin tissue in fetal and postnatal rats. Noting that extramedullary chromaffin tissue atrophied soon after birth (*i.e.*, when plasma glucocorticoid levels were falling) while intra-adrenal chromaffin tissue continued to thrive, he administered exogenous glucocorticoids to postnatal rats and observed that he could thereby prolong the survival of the extramedullary tissue (148). Lempinen's findings, and other observations on the effects of fetal decapitation on accumulation of epinephrine in the adrenal medulla (212), also supported the idea that the activity of the adrenal medulla depended upon cortical hormones.

In 1965, one of us (R.J.W.) became interested in the extreme sensitivity to circulating insulin often seen among patients suffering from pituitary failure (62). In such patients, plasma glucose concentration responds to the injection of insulin (or to the postprandial secretion of endogenous insulin) by falling to inappropriate low levels. Moreover, hypoglycemia may persist for hours; this leads to seizures and to pathological changes. This altered sensitivity to insulin had generally been attributed to the absence of hyperglycemic factors normally secreted from the pituitary (*e.g.*, growth hormone) or to the impairment in gluconeogenesis that might be expected to follow the loss of ACTH and the consequent decline in glucocorticoid secretion. However, this explanation did not seem adequate to explain the characteristic pattern of hypoglycemia observed in hypopituitary patients, since the length of time needed for circulating growth hormone or ACTH to raise plasma glucose levels was on the order of hours. It seemed more likely that, in addition to these pituitary hormones, hypopituitary subjects also lacked the substance or substances that normally act most rapidly to restore plasma glucose levels after the induction of hypoglycemia. Since epinephrine seemed the best candidate for this hypothetical fast-acting hyperglycemic agent, studies were initiated to determine whether pituitary failure also might interfere somehow with the synthesis or secretion of this catecholamine. It was known that epinephrine synthesis was catalyzed by PNMT (132), and that in adult mammals this enzyme was highly localized within the adrenal medulla (2). Hence, experiments were performed to determine whether hypophysectomy caused PNMT activity to decline in the rat adrenal (254). Subsequent studies, reviewed below, showed that, by suppressing glucocortocoid secretion, pituitary failure not only compromised epinephrine synthesis but also lowered epinephrine levels in the adrenal medulla (252, 254, 255) and the rate at which epinephrine was secreted in response to hypoglycemia (258). The large amounts of glucocorticoids needed to restore PNMT activity in hypophysectomized animals suggested at first that their effect on this enzyme lacked physiological significance. The full implications of this dosage requirement became clear, however, when the peculiarities of mammalian adrenal anatomy were taken into account: these doses were, in fact, quite compatible with the concentrations of glucocorticoids normally available to the medulla by virtue of its privileged location within the envelope of adrenal cortex.

In the few years that have elapsed since the discovery that glucocorticoids induced PNMT, recognition of this adrenocortical-adrenomedullary interaction has provided a useful general principle for explaining and predicting the behavior of the medulla under a variety of physiological circumstances. The following review describes the morphology and biochemistry of the adrenal medulla, the experimental evidence that its ability to synthesize epinephrine is controlled by glucocorticoid hormones, and the physiological evidence that this control mechanism actually operates in the normal mammal.

II. CHROMAFFIN CELLS IN THE ADULT MAMMAL

Chromaffin cells have been defined (55, p. xi) as neuroectodermal derivatives that stain positively with chromate salts (111), receive a preganglionic cholinergic innervation, and synthesize and store catecholamines. This latter biochemical property is shared with certain neurons in the brain and with postganglionic sympathetic neurons (251). In the adult mammal, chromaffin cells are largely confined to the adrenal medulla (55, pp. 76 and 237). Adrenomedullary chromaffin cells can, in general, synthesize both epinephrine and norepinephrine; extra-medullary chromaffin cells usually contain only norepinephrine. The term "chromaffin cell" was first used by Kohn in 1902 to characterize the cells' assumption of a brown color when stained with a chromic acid solution (143). This is the well-known chromaffin reaction. Cells storing such other monoamines as dopamine and serotonin [*e.g.*, mast cells and enterochromaffin cells (15)] can give falsely positive chromaffin reactions.

A. Histology and innervation

The chromaffin cells of the adrenal medulla are irregularly shaped and epithelioid; they are aggregated as groups, often in association with vascular sinusoids. Chromaffin cells vary in size, shape, and arrangement in different species. In addition to chromaffin cells, ganglion cells, single or grouped, are also found in the medulla (53, 236, 237, 260). Extra-adrenal chromaffin cells are also usually found in association with blood vessels (239).

The chromaffin reaction as used initially demonstrated cells storing both adrenal catecholamines. Not until 1953 were methods developed that allowed the selective staining of norepinephrine-containing cells. The first such technique was the potassium iodide method of Hillarp and Hokfelt (113). This and the histochemical fluorescence method of Eränkö (73) made possible the mapping of medullary distribution of epinephrine- and norepinephrine-storing cells. More recently, the glutaraldehyde reaction introduced by Coupland *et al.* (59), has been used for differential staining of the two cells types. This method is based on the reaction of glutaraldehyde with norepinephrine to form an insoluble complex; the product formed with epinephrine is more soluble and is lost during the subsequent processing of the tissue (57). The glutaraldehyde method has also been adapted to allow the two types of catecholamine cells to be differentiated by electron-microscopic examination (59).

When viewed with the electron microscope, medullary cells contain char-

acteristic secretory granules (the "chromaffin granules") in addition to the usual cell organelles (54, 154). The adrenal medullary catecholamines are stored in these granules (5, 12); they also contain dopamine β-oxidase, an enzyme involved in catecholamine synthesis (155). The chromaffin granules are round to oval electron-dense bodies, 1500 to 3000 Å in diameter (54, 154, 199) and are bounded by a unit membrane approximately 75 Å thick (54, 199). They appear to fill most of the cytoplasmic volume of the chromaffin cells. After glutaraldehyde fixation, epinephrine and norepinephrine cells can be differentiated by the appearance of their chromaffin granules (54, 59). Norepinephrine-storing granules are very electron-dense, and their cores tend to be eccentrically located and nonhomogeneous. The core of the epinephrine granule is not especially dense and is usually in the center of the granule. Epinephrine-storing cells have a larger number of secretory granules per cell than do norepinephrine cells (199). In some vertebrates (*e.g.*, the snake) norepinephrine granules tend to be larger than those storing epinephrine (242).

The adrenal medulla receives a preganglionic cholinergic innervation, primarily from the greater thoracic splanchnic nerves. Some minor fibers to the medulla also originate in the lesser thoracic splanchnic nerves or pass to it from the first or second lumbar ganglia (64, 174, 261). Most of the fibers supplying the adrenal glands are nonmyelinated (51, 52, 93). After traversing the cortex, the

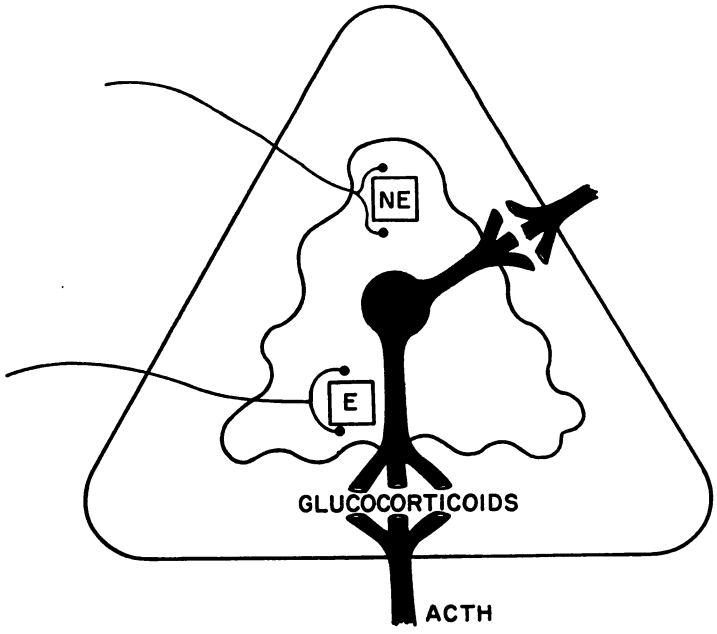

FIG. 1. Schematic diagram illustrating the anatomical relationship between the adrenal cortex and medulla, and the intramural adrenal portal vascular system. An "epinephrine cell" is shown lying near an adrenal sinusoid and a "norepinephrine cell" at some distance from the sinusoid. Both receive a preganglionic cholinergic innervation.

nerve fibers pass among chromaffin cells, running adjacent to large blood vessels (52, 154); they ultimately form coarse plexi from which the finest nerve fibers emerge to pass between adjacent chromaffin cells. Terminal boutons from these fibers apparently form true synapses with chromaffin cells (fig. 1) (51, 52). The presynaptic structure contains a variable number of synaptic vesicles and some mitochondria (51, 52, 103, 175); these vesicles are about 500 in Å diameter and are electron-clear; a few larger, electron-dense vesicles (about 1000 Å in diameter) can also be found (51, 52). The plasma membranes of the terminal bouton and its target chromaffin cell frequently show an increased electron density in the zone of contiguity; this pattern is thought to correspond to the pre- and post-synaptic thickenings associated with synapses between two neurons (51, 52, 103, 175). The synaptic endings on toad chromaffin cells apparently exist in two varieties that can be distinguished by the appearance of their synaptic vesicles (197).

Studies of adrenals taken from rats (112) and cats (171) in which the nerves to the adrenal had been transected at various levels suggest that there is a segmental distribution to the medullary innervation. On the basis of such evidence, Hillarp (112) postulated the existence of neuroeffector units with overlapping fields of influence. More recently, Marley and Prout (171) found that the three

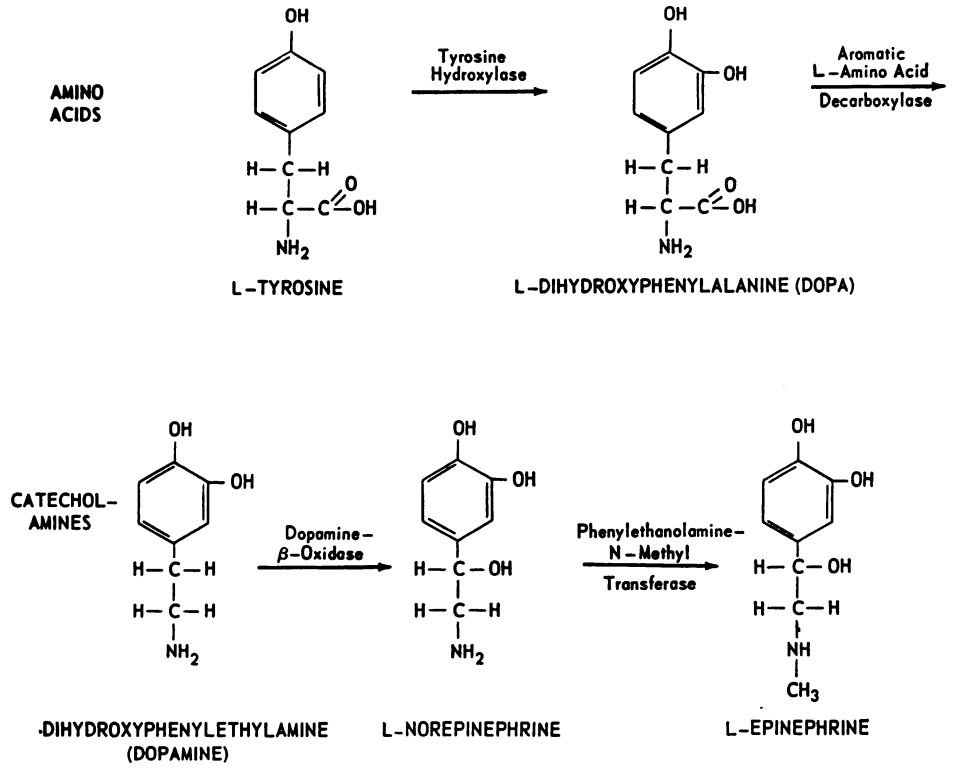

FIG. 2. Biosynthesis of catecholamines within adrenal chromaffin cells.

splanchnic nerves in the cat do not end in a plexus but innervate separate groups of chromaffin cells with relatively little overlap.

B. Catecholamine synthesis

Catecholamines are synthesized in chromaffin cells and also within postganglionic sympathetic neurons and certain brain neurons (fig. 2). Catecholamine biosynthesis is initiated by the uptake of circulating *l*-tyrosine into these cells. Plasma and tissue tyrosine levels exhibit a characteristic diurnal variation, human plasma levels rising almost 2-fold between 2 A.M. and noon (259). Such variation in availability of the precursor conceivably could influence the rate at which catecholamines are synthesized.

The enzyme tyrosine hydroxylase converts tyrosine to the catechol amino acid *l*-dihydroxyphenylalanine (*l*-dopa) (127, 185). Under most physiological conditions, tyrosine hydroxylation is probably the rate-limiting step in the synthesis of dopamine and norepinephrine (156), at least in postganglionic sympathetic neurons. The experimental evidence supporting this hypothesis includes the following: (a) Raising endogenous norepinephrine levels by administration of a monoamine oxidase inhibitor resulted in decreased synthesis of ^{14}C-norepinephrine from ^{14}C-tyrosine; no such effect was observed when the tyrosine hydroxylase step was bypassed by using ^{3}H-dopa as the precursor (227). (b) Denervation of the rat submaxillary gland resulted in declines in both norepinephrine content and tyrosine hydroxylase activity (218). (c) Weiner and Rabadjija (245) have shown that the physiological processes that increase or decrease the hydroxylation of tyrosine *in vivo* also cause parallel changes in the synthesis of ^{14}C-catecholamine from ^{14}C-tyrosine. (d) Decreases in the levels of endogenous brain norepinephrine after electroconvulsive shock treatment are associated with increased tyrosine hydroxylase activity in brain homogenates (184). Fewer data are available on the control of norepinephrine synthesis within the adrenal medulla. It seems possible that the conversion of dopamine to norepinephrine could also be limiting in this organ under appropriate conditions.

Aromatic *l*-amino acid decarboxylase catalyzes the conversion of *l*-dopa to the catecholamine dopamine (10, 88, 160). This enzyme is present in most mammalian tissues in large amounts (160). It catalyzes the decarboxylation of a relatively large number of physiological substrates (160). Norepinephrine is formed by the addition of a hydroxyl group to the β-carbon of dopamine (155). This reaction is catalyzed by an enzyme, dopamine β-oxidase (100, 131, 155), that is localized within chromaffin granules (100, 155) and is released from them along with catecholamines, ATP, and chromogranin (another granular protein) during medullary secretory activity (11, 67, 133).

Epinephrine synthesis involves the transfer of a methyl group from S-adenosylmethionine to the amine nitrogen of norepinephrine. The reaction is catalyzed by the enzyme phenylethanolamine-N-methyl transferase, PNMT (132), which is discussed in detail below.

The rates at which catecholamines are synthesized within the adrenal medulla probably can be controlled at several loci. Norepinephrine synthesis appear to

be controlled at the step of tyrosine hydroxylation: tyrosine hydroxylase activity is inhibited both *in vivo* (230) and *in vitro* (235) by norepinephrine, and Udenfriend and his associates have suggested that this end-product inhibition causes minute-to-minute adjustments in norepinephrine synthesis to accommodate changes in the release of the catecholamine (227). The rate of epinephrine formation appears to be controlled by PNMT activity. This methylating enzyme can be inhibited *in vitro* by epinephrine concentrations similar to those normally present in the adrenal(46). Hence, the minute-to-minute regulation of epinephrine biosynthesis, like that of norepinephrine, may also involve end-product inhibition. As described below, the PNMT activity and, probably, the level of PNMT protein within any chromaffin cell depend upon the concentration of glucocorticoids perfusing that cell. The medulla responds to changes in glucocorticoid perfusion over a period of hours, not minutes. Under normal circumstances, both adrenal tyrosine hydroxylase and PNMT probably function in the presence of saturating concentrations of their substrates, tyrosine and norepinephrine.

Isotopically labeled epinephrine has been recovered from the adrenals after administration of the following radioactive precursors: phenylalanine (109, 215, 233, 234), tyrosine (181, 233, 234), dopa (68, 233), dopamine (181, 246), and norepinephrine (26, 173, 233).

C. Phenylethanolamine-N-methyl transferase (PNMT)

In 1949 Bülbring demonstrated that homogenates of cat or dog adrenals could convert norepinephrine to epinephrine (24, 26). ATP and choline were also required for this reaction (26) but some methylation of added norepinephrine could occur even in the absence of added ATP; this phenomenon was attributed to the high levels of endogenous ATP within the homogenates. At about the same time Keller and Boisonnas demonstrated that methionine could serve as the methyl donor (125, 130) in epinephrine biosynthesis. Isotopically labeled epinephrine was found in the adrenals of rats fed ^{14}C-methionine for 4 days (125). Animals kept for 2 weeks on a diet deficient in methionine and choline had less epinephrine remaining in their adrenals after an insulin stress than did rats on a normal diet (130). The methylation of norepinephrine *in vitro* was found to be oxygen dependent (83, 102, 234). If methionine was used as the methyl donor *in vitro*, ATP and Mg^{++} were also necessary for methylation (132).

With beef adrenals, Kirshner and Goodall (132) identified and partially purified the norepinephrine-methylating enzyme in 1957. This enzyme was shown to be present in the supernatant portion of adrenal homogenates, and S-adenosylmethionine was the best methyl donor (132). The enzyme was further characterized by Axelrod (2), who termed it phenylethanolamine-N-methyl transferase (PNMT) on the basis of its substrate specificity; the enzyme could catalyze the N-methylation of any phenylethanolamine derivative, including such naturally occurring amines as norepinephrine, normetanephrine, and octopamine (2). In its presence a second N-methyl group could be added to epinephrine and metanephrine. Phenylethylamines were thought not to be

substrates, whereas the best naturally occurring substrates appeared to be normetanephrine and octopamine (2). More recent investigations on bovine medullas have shown that norepinephrine has an even lower Km (5×10^{-5} M *versus* 7.3×10^{-5} M for normetanephrine) and thus is most likely the preferred substrate for the enzyme (90, 137). PNMT activity can be inhibited by both its substrate, norepinephrine, and its product, epinephrine (91, 137); this inhibition is noncompetitive (91). Fuller and Hunt (91) point out that the concentrations of epinephrine found in the adrenals of the three species they investigated are far greater than the Ki for the inhibition of PNMT by epinephrine. Thus, if only 1 % of the adrenal epinephrine were unbound in the cytoplasm of the chromaffin cells, it would be sufficient to cause some inhibition of PNMT (91). PNMT has been reported by one observer (2), but not by another (90), to catalyze the N-methylation of *l*-norepinephrine to a greater degree than the *d* isomer. Its pH optimum in monkey adrenals, in 0.1 M phosphate buffer, was between 7.5 and 8.7 (132).

R. W. Fuller, B. J. Warren, and B. B. Molloy have recently observed (unpublished observations, 1970) that a nonphenylethanolamine, β-amino,3,4-dichlorophenylethylamine, can be a substrate for rat adrenal PNMT. The Km for this compound (4.5×10^{-6} M) is actually lower than that of norepinephrine.

PNMT appears to be a sulfhydryl-containing enzyme: 10^{-5} M *p*-chloromercuribenzoate almost completely inhibits enzyme activity (46, 137). The enzyme can also be inhibited by heavy metal ions (24). Cyclopropylamines such as tranylcypramine are potent competitive inhibitors of PNMT *in vitro* (144); no compounds have been described, however, that produce a significant inhibition of PNMT *in vivo*. By measuring the initial reaction rates of purified PNMT at various concentrations of norepinephrine and S-adenosylmethionine, Connett and Kirshner (46) have obtained evidence that PNMT is a two-substrate enzyme, with a tendency to bind S-adenosylmethionine preferentially over norepinephrine; its molecular weight was estimated as 38,000.

Although adrenal PNMT activity is found mainly in the $100,000 \times g$ supernatant (2), Goldstein and coworkers (98, 99) have reported that the enzyme is also found in the particulate fraction of adrenal homogenates. Some physicochemical properties of this particulate enzyme from a pheochromocytoma (99), *e.g.*, its thermolability, were found to be similar to those described for the norepinephrine-methylating enzyme in frogs (257). Since the frog enzyme had been shown not to be induced by glucocorticoids (257), the authors suggested that the existence of this enzyme in certain extra-adrenal chromaffin tumors might explain the ability of these tumors to synthesize epinephrine in the absence of an adrenocortical envelope (98). This hypothesis is discussed in greater detail below. Further purification of the soluble PNMT from mammalian adrenals indicated that at least two isozymes probably exist (99).

III. THE MAMMALIAN ADRENAL

A. Evolution of the adrenal medulla

Most of the chromaffin cells in the adult mammal are grouped together to form the adrenal medulla. The medulla is surrounded by another organ, the adrenal

cortex, of different embryologic origin and different secretory properties. This intimate association between cortical and chromaffin cells is not characteristic of all vertebrate species. In some lower vertebrates (*e.g.*, the dogfish), the two cells types are completely separate (48); as phylogenetic advances occurred, the two elements became more closely associated. The degree of association is reflected in the nature of the catecholamine stored by the chromaffin cells.

In fish, chromaffin and cortical cells are not juxtaposed. The main catecholamine stored by the chromaffin cells is norepinephrine (48), although variable amounts of epinephrine may also be present (13, 81, 82, 189, 190, 222, 247). Trams and Brandenburger Brown have reported the presence of epinephrine and of PNMT activity in the interrenal bodies of elasmobranchs (sharks and rays) (16, 232). These organs are homologous with the adrenal cortical cells of mammals. The authors interpreted their observations as suggesting that cortical cells in elasmobranchs may participate in the biosynthesis of catecholamines (16, 232).

Amphibians exhibit a closer relationship between cortical and chromaffin cells. In these animals, groups of chromaffin cells are interspersed among the steroid-producing cells, and both are imbedded within the mesonephros (6, 55, p. 229), the homologue of the mammalian kidney. The proportion of epinephrine to norepinephrine stored by the chromaffin cells varies among species; epinephrine comprises 31 to 47% of the total content (48, 120, 122, 190). Amphibians are unusual among vertebrates in that the chief catecholamine found in neurons is epinephrine and not norepinephrine (4, 79, 158). Bogdanski *et al.* (14) concluded that the widespread distribution of epinephrine in amphibians is unique to this vertebrate class. The properties of the PNMT found in both chromaffin cells and neurons of frogs suggest that this enzyme is a different protein from mammalian PNMT (257). Frog PNMT is discussed in greater detail below.

Chromaffin cells have a dual distribution in reptiles. Part of the chromaffin tissue lies outside the adrenal gland as a band closely applied to its surface; the remainder is distributed within the gland itself as small islets of tissue (159, 178, 245, 250). That the chromaffin cells outside the gland store only norepinephrine has been demonstrated by both histochemical and biochemical methods; cells inside the gland store predominantly epinephrine (9, 122, 243). PNMT activity was found to be very high in the intra-adrenal chromaffin tissue of the snake; in contrast, almost no enzyme activity could be discerned in the peripheral chromaffin tissue (256).

In birds, chromaffin and cortical cells are distributed apparently at random throughout the adrenal gland (74, 95). Norepinephrine has been reported to represent 55 to 70% of the total catecholamine present in the hen adrenal (74, 95, 220). Planimetric measurements showed that in the adrenals of various avian species norepinephrine cells constitute 50 to 100% of the total volume of chromaffin tissue (95).

In mammals, chromaffin cells have become grouped into a compact mass situated near the center of the adrenal gland. Cortical cells thus completely surround the chromaffin cell mass (the medulla), and their undiluted venous blood

selectively perfuses this tissue. There is considerable variation among species in the distribution and number of epinephrine- and norepinephrine-containing cells within the medulla. In rodents the ratio of the cross-sectional area of the cortex to that of the medulla is highest in the guinea pig and rabbit (1:40 and 1:60) (220). In these animals the main catecholamine found in the medulla is epinephrine, an average of 98% of the total (35, 115). A somewhat higher proportion of norepinephrine (1 to 18%) in the adrenals of rabbits was reported by some investigators (8, 36). In other rodents variable proportions of norepinephrine can be found in the adrenal, and the norepinephrine-storing cells form small groups or islets interspersed among the predominant epinephrine-storing cells. In rats, up to 15% of the cells store norepinephrine, and 9 to 20% of total catecholamine content determined biochemically is norepinephrine (20, 73, 76, 114). In mice 25 to 30% of the medullary cells store norepinephrine (20, 75, 76). In the hamster, norepinephrine cells are found on the periphery of the medulla along the border with the adrenal cortex (118, 193, 249, 260), and 8% of the total adrenal catecholamine is norepinephrine (247).

In contrast to the findings in rodents, proportionally more epinephrine cells tended to be located on the periphery of the cat medulla (216). Only 5% of the catecholamines extracted from the peripheral medullary tissue was norepinephrine, as opposed to 19% of the catecholamines extracted from central medullary tissue (216). The percentage of total adrenal catecholamines represented by norepinephrine is 38 to 41% for the cat (29, 80, 101, 220), 45 to 55% for the lion (101, 247), and 20 to 27% for the dog (220, 247). The ratio of the two substances in cat adrenals is extremely variable; as little as 8% or as much as 84% can be norepinephrine (29). The variability is diminished considerably if littermate animals are studied (32).

The distribution of the two types of chromaffin cells in ungulates is similar to that of the cat. In the cow (1, 219, 224), the ox (192, 193, 225), the horse (193, 225), the pig (225), and the sheep (225), epinephrine cells are most prevalent along the adrenocortical-medullary junction. Norepinephrine is 27 to 40% of total medullary catecholamines in the cow (101, 247), 20 to 26% in the horse and ox (247), 30 to 53% in the pig (101, 247), 33% in the sheep (247), and 33 to 39% in the gazelle (101).

In whales, 52 to 83% of adrenal catecholamine is norepinephrine (28, 204).

In primates, norepinephrine is absent [*e.g.*, in the baboon (101)] or is found in low concentrations [9% in *Macacus radiatus* (85)] and 6 to 8% in the macaque d'Algerie (8). In adult man it represents 9 to 22% of the medullary catecholamines (85, 220, 247).

Relatively little information is currently available about the comparative anatomy of the medullary circulation. The observation that chromaffin cells near the corticomedullary junction tend to contain norepinephrine in some species and epinephrine in others ultimately may be explained when detailed information becomes available on the intra-adrenal vascular patterns in each species. For example, in animals whose epinephrine cells are adjacent to the cortex, the portal venous blood may be distributed concentrically; in species whose epineph-

rine cells are concentrated within the center of the medulla, the portal blood distribution may be radial.

B. Vascular supply

Concomitant with the appearance of a distinct medulla, the adrenal glands of higher vertebrates also developed an intramural circulatory system (fig. 1). The mammalian adrenal gland is richly supplied by a number of arteries that enter the gland at different points. These arteries form a plexus in the capsule of the gland and give rise to an anastomosing sinusoidal network of cortical arteries that surrounds the cords of cortical parenchymal cells. The sinusoids become progressively wider and coalesce as they approach the center of the medulla (7, 110, 153). A few major arterial branches from the capsule penetrate the trabeculae of connective tissue and reach the medulla without perfusing the cortex. Once in the medulla, they branch repeatedly to form a capillary net around the groups of chromaffin cells (110). The medullary capillaries empty into the same venous system as that which drains the cortex; this system eventually forms the suprarenal vein (7, 110, 153). Endothelial cells lining the medullary vescular system exhibit prominent fenestrations (72), a feature also typical of these cells in other endocrine organs. The adrenal medulla thus has a rich dual blood supply. It receives blood that has drained the adrenal cortical cells and is rich in cortical steroids, and also has its own direct arterial supply. This arrangement may be important in determining the type of catecholamine synthesized and stored by individual chromaffin cells. The proportions of the total blood supply to given chromaffin cells that derive from the arterial capillary net and from the portal sinusoidal blood have not yet been examined.

IV. PNMT INDUCTION BY GLUCOCORTICOIDS

The first studies on adrenocortical control of PNMT activity were published in 1965, and showed that PNMT activity, like adrenal weight, declined markedly within several days of hypophysectomy (254). The fall in enzyme activity was proportionally greater than the decrease in adrenal weight, which was due primarily to the atrophy of adrenocortical tissue. The amount of epinephrine in the adrenals also decreased after hypophysectomy, as did the percentage of total adrenal catecholamine represented by epinephrine (114, 254). If hypophysectomized rats were given daily injections of ACTH for 6 days, the total activity of PNMT in adrenal tissue increased 3-fold, thereby returning to the normal range (254). The fraction of adrenal catecholamine represented by epinephrine responded similarly (114, 254).

Three possible mechanisms were initially suggested to explain the depressed enzymatic methylation of norepinephrine in the ACTH-deprived rat (254): (a) A methylating enzyme could have been present in the adrenal cortex. The activity of this enzyme would be expected to decline after hypophysectomy, and, like cortical size, to be restored by administration of ACTH. (b) ACTH could have exerted a direct effect on PNMT in the adrenal medulla. (c) ACTH could have enhanced medullary PNMT activity indirectly by stimulating glucocorti-

coid synthesis in the adrenal cortex and delivery to the medulla. To examine the first of these mechanisms, adrenal glands were dissected into cortical and medullary fragments, which were assayed separately for PNMT activity. Less than 10% of the total enzyme activity in the adrenal could be attributed to the cortex in either hypophysectomized or control animals (254, 255). To determine whether ACTH stimulated PNMT activity by acting directly on the adrenal medulla, experiments were performed in which plasma ACTH levels were raised or lowered pharmacologically by administration of methopyrapone, an inhibitor of glucocorticoid biosynthesis, or dexamethasone, a highly potent synthetic glucocorticoid, while glucocorticoid levels either remained constant or changed in the opposite direction (254, 255). In both experimental situations, PNMT activity did not change in the same direction as plasma ACTH levels, although adrenal weight (primarily a reflection of cortical mass) increased with methopyrapone and decreased with dexamethasone (254, 255). These observations indicated that the effects of ACTH on medullary PNMT activity were not direct and suggested that they were mediated by changes in glucocorticoid secretion.

The suspicion that ACTH elevated PNMT activity in hypophysectomized rats by stimulating glucocorticoid secretion was confirmed by treating hypophysectomized animals with dexamethasone for 6 days. This treatment, which caused no elevation in adrenal weight, completely restored PNMT activity (254, 255).

Long-term increases in the levels of circulating ACTH can elevate the PNMT activity in the adrenals of intact rats. Such elevations have been reported in animals bearing transplantable ACTH-secreting tumors (238) and in animals subjected to unilateral adrenalectomy (39). Moreover, such chronic stresses as drinking hypertonic salt solutions (37) and repeated immobilization (145, 146) also increase PNMT activity. Exposure of rats to continuous light or darkness results in decreased adrenal PNMT activity, probably because both environments lessen the amplitude of the daily rhythm in glucocorticoid secretion (200). Adrenal glands taken from hibernating ground squirrels contain less epinephrine than those from active animals, suggesting that PNMT activity also falls during hibernation (68). An increase in adrenal epinephrine in hibernating hedgehogs has also been reported (229). The basis of this apparent contradiction has not been determined. The alterations in adrenal epinephrine content and PNMT activity reported to occur after a variety of treatments are summarized in table 1.

A. Hormonal specificity

The specificity of PNMT elevation by ACTH was examined in groups of hypophysectomized rats treated with partially purified preparations of each of the six major pituitary hormones (252). Administration of ACTH caused a 48% increase in adrenal weight and a 160% rise in PNMT activity (252). Doses of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) that produced significant increases in the weights of the ovaries and uteri had no effect on PNMT (252). Prolactin, thyroid-stimulating hormone (TSH), and growth hor-

TABLE 1

Hormone-induced alterations in epinephrine content and phenylethanolamine-N-methyl transferase (PNMT) activity of adrenal glands

Treatment	Animal Species	Epinephrine		Adrenal PNMT Activity	Reference
		Adrenal content	Excretion in urine		
Hypophysectomy	Rat	↓		↓	199, 201, 252, 254, 255
	Dog	↓		↓	258
	Lamb			↓	44
	Man		↓		161
	Frog			=	257
Hypophysectomy + ACTH or steroids	Rat	↑		↑	199, 201, 252, 254, 255
	Dog	↑		↑	258
ACTH or hydrocortisone	Newborn rat	↑		↑	92, 210, 211
	Newborn rabbit	↑		↑	210, 211
Adrenal medullary explants + steroids	Rat	↓		↓	198
		↑		↑	198
Estrogen + aldosterone	Guinea pig	↑			163
+ growth hormone		↑			
+ glucagon		↑			
Insulin	Rat			↑	134–136
Glucagon	Rat			↑	134
Aminogluthetamide	Rat			↑	69
Unilateral adrenalectomy	Rat			↑	39
Hypothalamic stimulation	Cat			↑	207
Immobilization stress	Rat	↑		↑	145, 146
Drinking of saline	Rat			↑	37
Continuous light or darkness	Rat	↓		↓	200
Hibernation	Hedgehog	↑			229
	Ground squirrel	↓			68

mone also had no effect on PNMT activity, although growth hormone caused a significant rise in body weight (252).

Several experiments were performed to determine whether glucocorticoids alone among steroid hormones were able to induce PNMT activity. In an initial study, large doses of compounds with the physiological characteristics of four families of steroid hormones (estrogens, androgens, mineralocorticoids, and glucocorticoids) were tested for their ability to restore epinephrine-forming activity after hypophysectomy (252). Estradiol and testosterone had no effect on PNMT activity, but treatment with aldosterone (50 μg/day) and dexamethasone (1 g/day) led to 50% and 195% increases, respectively (252).

The structure-activity relationships of steroids that induce PNMT activity were further investigated with the use of various progesterone derivatives hydroxylated at the 11-, 17-, or 21-position (201). Hydrocortisone, which contains

all three hydroxyl groups, was the most potent natural steroid. The increase in activity conferred by 11-β-hydroxylation was about as great as that conferred by 17- or 21-hydroxylation (201). This pattern contrasts with that of another steroid-inducible enzyme, glutamic synthetase, for which 11-β substitution appears to be of special importance (182). The ability of a progesterone derivative to induce PNMT was also roughly proportional to its solubility in water (201).

Prolonged treatment (4 months) of guinea pigs with estrogens was reported to cause hyperplasia of adrenal medullary cells and an increase in adrenal epinephrine content (163). The effects were magnified if animals were treated concurrently with either aldosterone or growth hormone (163). These findings are difficult to relate to current concepts about the specific enzymes controlling catecholamine biosynthesis and might be the consequence of nonspecific metabolic imbalances. No such effects have been reported after brief periods of estrogen treatment in rats (252).

Both insulin and glucagon have been reported to stimulate PNMT activity in rat adrenals (134–136). The stimulation was evident within 6 hours after a single dose of either hormone. Daily administration of 6 units of insulin for 6 days resulted in a 28% increase in PNMT activity (136). When insulin was administered to hypophysectomized rats along with ACTH, the resulting increase in PNMT activity was somewhat larger than that produced by ACTH alone (136). It is possible that the insulin effect results not from a change in the amount of enzyme protein but from a decrease in the end-product inhibition of PNMT activity concomitant to the depletion of adrenal catecholamines.

Aminoglutethimide, a drug that suppresses glucocorticoid secretion from the adrenal cortex, recently has been reported to increase PNMT activity (69). This surprising finding may be related to a rebounding stimulation of corticoid secretion (aminoglutethimide acts only briefly) or perhaps to another biochemical action of the drug.

B. Dose-response characteristics

In the initial studies described, the doses of dexamethasone used to induce PNMT were much higher than those generally administered to rats. This fact cast doubt on the physiological significance of their effect. Experiments therefore were performed to define the precise dosage requirements for PNMT induction and to compare them with those necessary for other glucocorticoid actions. Hypophysectomized rats were treated for 4 days with various doses of hydrocortisone, corticosterone, or dexamethasone (252). Animals given either natural steroid (2 mg/kg) showed no change in PNMT activity. (This dose is generally considered sufficient to replace endogenous glucocorticoids in adrenalectomized animals (195).) Three milligrams of hydrocortisone per rat per day produced a slight rise (24%) in epinephrine-synthesizing ability, and 30 mg of hydrocortisone per day caused a 74% rise. The increase in PNMT produced by the largest dose of hydrocortisone was only about half as great as that caused by 1 mg of dexamethasone (252). In contrast to the very high dosage requirements for glucocorticoids, the amount of ACTH needed to restore PNMT activity in hypoph-

ysectomized rats was no greater than the dose needed to maintain adrenal weight (252). PNMT activity was therefore much more responsive to exogenous circulating ACTH than to injected glucocorticoids. Since the effect of ACTH on PNMT previously had been shown to be indirect and to be mediated by glucocorticoid secretion, PNMT activity appeared to be controlled not by the glucocorticoid concentrations in the general circulation but by those present within the adrenocortical venous blood that perfuses the medulla. The concentration of steroids in the adrenal venous effluent is at least 100-fold greater than that found in the general circulation (142). Glucocorticoid concentrations in the blood leaving the adrenal cortex are probably even greater, since this blood has not yet been diluted with that from the arteries to the adrenal medulla. Hypophysectomy depresses the concentration of the steroid in the adrenal veins and peripherally. Both concentrations can be restored to normal by administration of ACTH, but only peripheral blood levels are restored by customary doses of glucocorticoids. The expected effects of hypophysectomy and various hormones on the concentrations of glucocorticoids in plasma and adrenal venous blood are illustrated diagrammatically in figure 3.

On the basis of this formulation, we anticipated that ACTH doses influencing peripheral target organs in hypophysectomized animals to the same extent as did a given dose of hydrocortisone would be considerably more potent in stimulating epinephrine biosynthesis. To test this hypothesis, hypophysectomized animals were treated with several dosage levels of ACTH or hydrocortisone, and the effects of the hormones on splenic weight and adrenal PNMT were measured (252). It should be recognized that splenic weight is influenced by many factors, of which the concentration of glucocorticoids in the general circulation is but one (180). The weight of the spleen was depressed equally by 10 U of ACTH or

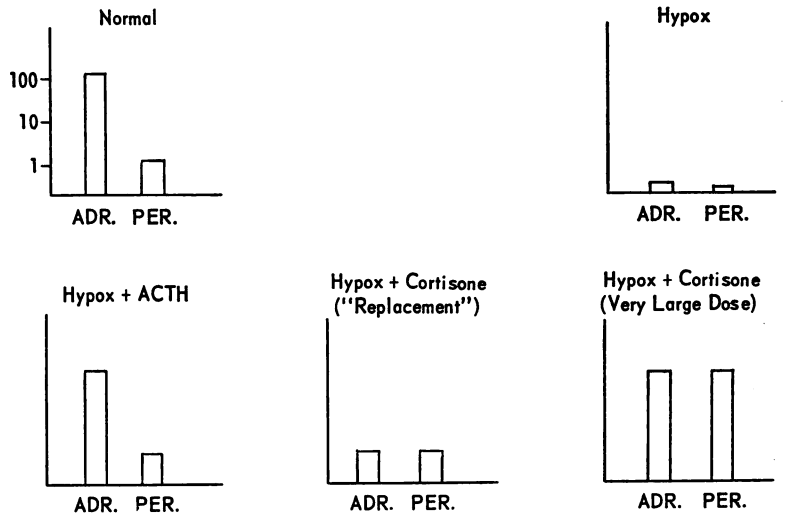

FIG. 3. Schematic diagram showing anticipated effects of hypophysectomy and various hormonal treatments on the concentrations of glucocorticoids in adrenal venous blood and peripheral venous blood. Concentrations are given as arbitrary units.

3 mg of hydrocortisone. However, 1 U of ACTH was as active as 30 mg of hydrocortisone in restoring PNMT activity. Hence, ACTH was about 100 times more potent in enhancing PNMT activity in the hypophysectomized rat than it was in depressing splenic weight. These findings tended to confirm the hypothesis that the very high concentrations of glucocorticoids present in the medullary sinusoids are vital in the maintenance of PNMT activity.

In another experiment, adrenal medullary cells in rats were removed from their natural envelope of adrenal cortex and transplanted to the anterior chamber of the eye. Exposure of the cells for 6 days to the glucocorticoid levels present in the peripheral circulation resulted in a marked fall in PNMT activity (from 12.87 ± 2.00 to 0.22 ± 0.11 μmoles/mg of protein/hr); also, epinephrine disappeared in the explanted tissue (198). Low doses of dexamethasone (0.2 mg/day for 6 days) caused the activity of hepatic tyrosine transaminase, another steroid-inducible enzyme, to rise in rats bearing the medullary explants but had no effect on the PNMT activity of the explanted tissue (198). A 1-mg dose of dexamethasone caused a substantial increase in PNMT activity (from 0.22 ± 0.11 to 2.38 ± 0.35 μmoles/mg of protein/hr), as well as the appearance of epinephrine in the explants, yet caused no further increase in tyrosine transaminase activity (198). Other investigators have shown that a single dose of 0.5 mg of cortisol increases tyrosine transaminase activity manyfold (214). This dose is considerably lower than the minimal dose needed to elevate PNMT activity in hypophysectomized rats.

C. Possible mechanisms of increased PNMT activity

The addition of hydrocortisone or dexamethasone to adrenal homogenates from normal or hypophysectomized rats has no direct effect on PNMT activity (255); hence the mechanism by which the glucocorticoids act appears not to involve enzyme activation. The slow time-course of PNMT induction in hypophysectomized rats (255) also suggests that the increase in enzymatic activity reflects either an increase in the synthesis of new enzyme or a slowing of its catabolism.

To examine the possibility that glucocorticoids enhance PNMT activity by stimulating the synthesis of new enzyme protein, dexamethasone was administered to groups of hypophysectomized rats with or without actinomycin D or puromycin pretreatment (255). One group was given a 4-hr intravenous infusion of 1 mg of dexamethasone per hour and was killed 5 hr later. This short treatment produced a small but significant increase in PNMT activity (255). Other groups of rats were treated with actinomycin D (1 mg/kg of body weight intravenously) or puromycin (40 mg/kg of body weight intravenously); some rats in each group were then given a similar infusion of dexamethasone. Neither actinomycin D nor puromycin altered the basal activity of the epinephrine-forming enzyme in the adrenal medulla (255). Both agents, however, blocked the rise in enzymatic activity caused by dexamethasone. These results are compatible with the hypothesis that glucocorticoids elevate PNMT activity by increasing the rate of synthesis of the enzyme protein.

Coupland and MacDougall (56, 58) observed that addition of hydrocortisone

(10 μg/ml) to tissue cultures of rabbit extra-adrenal chromaffin cells caused these cells to begin to accumulate epinephrine. Since the cells normally contain only norepinephrine the data can be interpreted as indicating that the chromaffin cells responded to the steroid by synthesizing new enzyme. The addition of deoxycorticosterone to the culture media had no effect (56, 58).

D. Effects of hypophysectomy and glucocorticoids on adrenal epinephrine content and epinephrine secretion

Removal of the pituitary gland is followed by a gradual fall in the epinephrine content of the adrenals; this decline continues for at least 50 days (252). The half-life of epinephrine disappearance in the adrenals of hypophysectomized rats is on the order of 30 days; this rate is somewhat slower than the 7-day half-life in normal animals (estimated isotopically) (233) and suggests that some epinephrine continues to be synthesized after hypophysectomy. Hypophysectomy is also associated with a slight increase in adrenal norepinephrine content. This increase is not stoichiometric with the decrease in epinephrine levels. Injection of ACTH or dexamethasone to hypophysectomized rats restores the epinephrine levels in the adrenals to normal (252).

To determine whether the secretion of epinephrine is also impaired after hypophysectomy, we assayed adrenal venous blood from chronically hypophysectomized dogs for epinephrine and norepinephrine basally and after the induction of hypoglycemia with insulin (258). Among unoperated control dogs, basal epinephrine secretion averaged 7.5 ± 0.01 ng × gland^{-1} × min^{-1} per kg of animal weight and rose to a peak of 31.9 ± 6.1 ng × gland^{-1} × kg^{-1} × min^{-1} in response to insulin-induced hypoglycemia. The proportion of total blood catecholamines represented by epinephrine was 82 ± 4.2%. In contrast, among hypophysectomized dogs, basal epinephrine secretion averaged 4.2 ± 0.7 ng × gland^{-1} × kg^{-1} × min^{-1} and rose to a peak of only 15.6 ± 4.9 ng × gland^{-1} × kg^{-1} × min^{-1} after insulin (258). The proportion of total blood catecholamine represented by epinephrine, 62.2 ± 8.3%, was significantly lower in these dogs than in control animals. Treatment with ACTH restored the basal rate of epinephrine secretion to normal in hypophysectomized animals. Moreover, the peak concentration of adrenal venous epinephrine after the injection of insulin was elevated beyond that seen in unoperated control dogs (to 74.4 ± 24.5 *versus* 31.9 ± 6.1 ng × gland^{-1} × kg^{-1} × min^{-1}). These studies indicated that the pituitary and adrenal cortex also control PNMT activity in the dog. They further revealed that the decline in epinephrine synthesis observed in hypophysectomized mammals is, in fact, associated with impaired secretion of the catecholamine in response to one of its major physiological stimuli, hypoglycemia.

E. Other effects of hypophysectomy on the adrenal medulla

1. Other enzymes involved in catecholamine synthesis and metabolism. Hypophysectomy causes decreases in the activities of tyrosine hydroxylase, monoamine oxidase, and catechol-O-methyltransferase in the whole rat adrenal (255). Like adrenal weight, the activities of these enzymes are not restored by dexamethasone treatment.

2. Morphological changes in the adrenal medulla after hypophysectomy. In the adrenals of intact rats, norepinephrine cells appear after glutaraldehyde-osmium tetroxide fixation as small, dark islets scattered, apparently at random, among the colorless epinephrine-storing cells (59, 199). No remarkable changes are noted in the distribution or relative size of the norepinephrine-cell islets until 50 days after hypophysectomy. At this time the islets appear larger and are prominent along a conspicuous concentric ring at the border between the adrenal cortex and medulla (199). A small decrease in the size of the rat adrenal medulla, estimated planimetrically, has been observed after hypophysectomy (177).

At the ultrastructural level, hypophysectomy causes a decline in the number of granules in each epinephrine-storing cell, and the cells take on a vacuolated appearance (199). In the first week after hypophysectomy, relatively few cells have an altered appearance. The granules in the affected cells are larger and their central electron-dense core becomes less compact, sometimes enclosing fine fibrillar material. A few cells display cytoplasmic vacuoles. At 34, 40, or 50 days after surgery, abnormal granules fill most of the cytoplasm of many cells (199). Moreover, morphologic signs of synthetic hyperactivity are notable among the most depleted cells; the cisternae of the rough endoplasmic reticulum appear vesicular and increase in number, and the Golgi apparatus becomes hyperplastic, ultimately surrounding the nucleus (199).

At all times after hypophysectomy, the adrenal contains epinephrine cells of normal ultrastructural appearance interspersed among other cells showing signs of marked depletion of secretory granules. Seventeen days after hypophysectomy, some cells contain mixed granule populations, *i.e.*, granules exhibiting the characteristics of both epinephrine- and norepinephrine-storing organelles. These results tend to corroborate the histochemical finding that in chronically hypophysectomized animals some epinephrine-storing cells begin to accumulate norepinephrine. The concentric ring of norepinephrine cells next to the adrenal cortex in hypophysectomized animals suggests a gradient in the steroid concentrations available to medullary cells (199).

Treatment of hypophysectomized rats with either dexamethasone or ACTH completely restores to normal the light- and electron-microscopic appearance of the medullary chromaffin cells (199).

F. Inducibility of PNMT isozymes

In the frog, epinephrine (4, 79, 158) and PNMT activity (257) are widely distributed; enzyme activity has been found in the brain, heart, and spleen (257) as well as in the adrenal. Since these organs lack an adrenal cortex, studies were performed to examine the dependence of frog PNMT upon high concentrations of glucocorticoids and to characterize the physicochemical characteristics of the frog enzyme. After hypophysectomy, no decline was found in the activity of PNMT within any organ studied, even though adrenal corticosterone content declined significantly (257). The physicochemical properties of frog PNMT differed from those of the rat enzyme in several ways: frog enzyme was found to be thermolabile at 47°C, was as active catalytically at 30°C as at 38°C, and was relatively immobile on starch-block electrophoresis at pH 8.6 (3, 257). The sub-

strate specificity of frog PNMT was similar to that of the rat enzyme, and both enzymes were largely confined to the cytoplasm (3, 257).

G. Extra-adrenal PNMT and glucocorticoids

1. Fetus. In the fetus, relatively large amounts of chromaffin tissue exist outside the adrenal medulla, especially along the aorta and the sympathetic nervous chain (47, 49, 50). The largest collection of extra-adrenal chromaffin cells forms the organ of Zuckerkandl (262). Most extra-adrenal chromaffin cells degenerate and disappear rapidly within the first 2 weeks of birth in rats (22, 147, 148, 240) or during the first 3 years of life in man (106, 124, 248). Administration of cortisone or hydrocortisone not only prevents postnatal degeneration of extra-adrenal chromaffin cells (147, 148, 151) but can actually unbalance the size and number of new chromaffin cells (148). This action probably involves stimulation of the differentiation of pheochromoblasts into chromaffin cells (148); the effect on extra-adrenal chromaffin tissue is dose-dependent. Small doses of hydrocortisone only prevent the normal involution of the cells, but larger doses produce a progressive increase in the volume of the tissue (148). The steroid effect is also time-dependent (148). The earlier after birth that the treatment is begun, the more effective it is (147, 148). Large doses of ACTH also prevent the disappearance of extra-adrenal chromaffin cells but large doses of deoxycorticosterone have no such effect (147, 148, 151). Lempinen (148) suggested that the normal involution of extra-adrenal chromaffin tissue is due to a decline in the level of cortical hormones associated with birth. Thyroidectomy has no effect on the perinatal development of extra-adrenal chromaffin tissue (150).

Extra-adrenal chromaffin tissues of various animal species synthesize and store norepinephrine (22, 221) both pre-and postnatally. A few observations suggest that they might also store minute amounts of epinephrine (106, 124, 187, 188, 221, 248) and contain trace levels of PNMT activity (94, 187, 211, 213). In a comprehensive study of the paraganglia of the rabbit, Brundin (22) found that 70% of the norepinephrine was sedimentable, about the same proportion as found in the adrenals at the same age. Electron-microscopic examination of the cells revealed electron-dense granules in the cytoplasm similar to those found in the medullary cells (22, 240). The synthesis and turnover of norepinephrine formed from ^{14}C-tyrosine or ^{14}C-dopa was slower than that in the medullary cells (22).

Histochemically, treatment of newborn rats with hydrocortisone for 9 days increases the chromaffin reaction and decreases formalin-induced fluorescence; this suggests an increase in the total catecholamine content of the tissue and enhanced conversion of norepinephrine to epinephrine (149). The appearance of epinephrine in the organ of Zuckerkandl after hydrocortisone treatment has been demonstrated by thin-layer chromatography (77, 78, 149) and chemical assay (213). Epinephrine and PNMT activity were present in the extra-adrenal chromaffin tissue of 9-day-old rats that had been treated with hydrocortisone for the first 7 days of life (211, 213). Adrenalectomy of the mother between days 16 and 17 of pregnancy resulted in a slight increase in the chromaffin reaction of extra-

adrenal chromaffin tissue from the young; hydrocortisone substitution inhibited the effect of adrenalectomy, suggesting that maternal steroid might inhibit fetal ACTH secretion (152). The chromaffin reaction was negative in the extra-adrenal chromaffin tissue of fetuses whose mother had been hypophysectomized between days 16 and 17 of pregnancy (152). Fetal hypophysectomy produced no such effect.

In the rabbit, part of the extra-adrenal chromaffin tissue is continuous with the chromaffin cells of the adrenall medulla (49). Interestingly, norepinephrine is found in the chromaffin cells located outside the gland, but only epinephrine is found in the chromaffin cells of the medulla (56, 58). When the extra-adrenal chromaffin cells were grown *in vitro*, in the presence of hydrocortisone or corticosterone concentrations comparable to those found in adrenal venous blood, epinephrine was synthesized and stored in the tissue (56, 58). Some of the cells even began to accumulate granules with ultrastructural characteristics of epinephrine cells (58).

2. Adult. In the adult mammal, most of the PNMT activity in the body is localized to the adrenal medulla. Low PNMT levels have been found in the heart (2) and in the brain (38, 202); enzyme activity is unequally distributed in the brain, with highest levels in the olfactory areas and the hypothalamus (202). Small amounts of epinephrine (5 to 15% of the total catechloamine content) have been identified in the mammalian brain with bioassay methods (241) and fluorescence techniques (107). The formation of epinephrine from intraventricularly administered ^{3}H-norepinephrine and ^{14}C-tyrosine has also been demonstrated in rats (202) and monkeys and cats (164).

H. Ontogenesis of PNMT in the adrenals, and the role of glucocorticoids

Norepinephrine is found in fetal rat adrenals at 16½ days of gestation (209–212) and in the rabbit at 19 days (21, 208, 210–212). Epinephrine is first detected at 17½ days of gestation in the rat and between 21 and 24 days in the rabbit (211, 212). The prenatal appearance of epinephrine follows the appearance of PNMT activity. PNMT can be measured in the rat adrenals after day 17½ of gestation; its activity rises sharply by day 20½ and continues to rise until birth (168). The rise in PNMT activity is preceded by a parallel increase in steroid synthesis by the adrenal cortical cells on days 16½ and 17½ of gestation (209). Since a large increase in the volume of the rat adrenal medulla also occurs between days 19½ and 20½ (128), Fuller and Hunt suggested that the rapid increase in PNMT activity taking place during this time reflected increasing medullary volume (92). Margolis *et al.* (168) showed, however, that the rise in adrenal PNMT activity actually began several days before the morphologic changes, that is, on day 17½ of gestation. In man, both catecholamines can be detected in fetuses weighing 42 g, the smallest thus far investigated (106).

The PNMT activity and epinephrine content of fetal adrenals can be modified experimentally. Decreases in both can be obtained by fetal decapitation or hypophysectomy during late pregnancy; both can be restored to normal levels by supplemental therapy with ACTH or glucocorticoids. Hypophysectomy of fetal

lambs at 93 to 105 days of gestation also causes impaired epinephrine synthesis in the adrenal medulla (44). Fifty days later, adrenal epinephrine content is only 56% of normal, even though total catecholamine content is normal (44). If rat fetuses are decapitated *in utero*, almost no PNMT activity is found in their adrenals at term and epinephrine levels are markedly reduced (92, 209, 211). The administration of a single dose of ACTH or cortisone to the decapitated fetuses or to the mother restores both PNMT and epinephrine (92, 211). Treatment of the mother with dexamethasone from day 12 to day 21 of pregnancy can also raise the PNMT activity and the epinephrine content of the adrenals of her young, examined at 21 days after birth (194). When the young are 45 days old, PNMT activity is no longer elevated, but epinephrine content is still above normal (194).

Steroid secretion of adrenal glands in the newborn rat is biphasic. It increases until 2 days of age and then is followed by a period of lowered steroid secretion (196). Thereafter the rate of steroid secretion again rises, attaining adult levels by 7 days of age (196).

The age at which medullary cells become innervated varies among species. In the lamb, innervation is complete before birth (41–43, 223); in the calf the process does not begin until 2 to 3 weeks of age (41–43, 223).

V. THEORETICAL RELATIONSHIP BETWEEN GLUCOCORTICOID CONCENTRATION AND PNMT ACTIVITY

On the basis of the evidence just described, one can represent the relationship between glucocorticoid levels in the blood that perfuses the adrenal medulla and the PNMT activity of the chromaffin cells by a sigmoid theoretical curve (fig. 4). Below a certain glucocorticoid concentration, synthesis of PNMT will not be

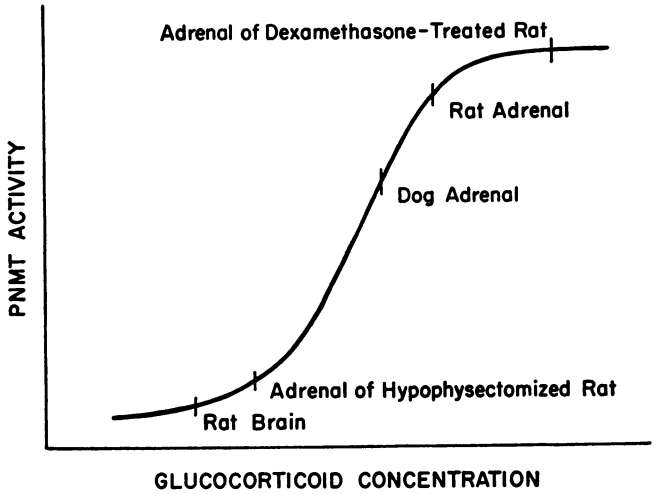

FIG. 4. Theoretical curve describing the relationship between the glucocorticoid concentration perfusing a tissue and its content of phenylethanolamine-N-methyl transferase (PNMT).

stimulated, and the enzyme will be present at "basal" levels of activity. (Indeed, these levels may represent the sum of even lower levels of the inducible "mammalian" form of PNMT plus the activity of an uninducible "frog" isozyme.) At a threshold concentration, the synthesis of PNMT begins to be stimulated, leading to an increase in steady-state enzyme activity. The threshold glucocorticoid concentration needed to elevate PNMT activity appears to be considerably above the range of concentrations normally present in the systemic circulation (139); it is, however, well within the range available to the medulla from the intra-adrenal portal vascular system. As intramedullary glucocorticoid levels continue to rise, steady-state PNMT activity shows a parallel response, probably manifesting a linear relationship over at least a portion of the theoretical curve. Ultimately, a point is reached at which further increases in glucocorticoid availability cause little or no additional increment in PNMT activity, and the curve levels off. This plateau could reflect maximal formation of polysomes coded for PNMT, the tendency of amino acid supply to become limiting (perhaps because of excessive gluconeogenesis), maximal suppression of PNMT catabolism, or a host of other biochemical mechanisms. Similar "input-output" theoretical curves could be drawn that relate adrenal epinephrine content to PNMT activity or display adrenal epinephrine content as a function of adrenal venous glucocorticoid levels.

In the linear portion of the curve, adrenal PNMT activity is neither at basal levels nor maximally stimulated; theoretically, the enzyme should respond to changes in glucocorticoid perfusion with approximately proportional changes in activity. If the rate at which PNMT activity attained its new steady-state level after a prolonged increase or decrease in glucocorticoid concentration were rapid, corticoids could properly be claimed to "regulate" epinephrine synthesis, and diurnal changes in adrenocortical secretion rates would be expected to produce parallel changes in PNMT levels. Whether this rate was fast or slow would depend primarily on the rate at which PNMT was itself catabolized [*i.e.*, enzymes that are catabolized slowly take a long time to attain new steady-states, once their synthesis is disturbed (98)]. The time-course of the rise in adrenal PNMT activity among hypophysectomized rats given dexamethasone is, in fact, very slow compared with other steroid-induced enzymes such as hepatic tyrosine transaminase or tryptophan pyrrolase. Moreover, repeated attempts to demonstrate a diurnal rhythm in PNMT activity have been unsuccessful. The apparent time-constants of the relationship described in figure 4 suggest that changes in glucocorticoid secretion influence PNMT activity only if they are chronic; these hormones probably do not regulate epinephrine synthesis on a minute-to-minute basis, at least in the animals examined to date.

It becomes of theoretical and perhaps clinical significance to determine where on figure 4 each mammalian species normally lies. The question can be approached experimentally in two ways: (a) animals can be injected with high doses of glucocorticoids and the extent of PNMT stimulation determined; or (b) endogenous glucocorticoid levels can be depressed by hypophysectomy or by administration of low doses of glucocorticoids, and the rate at which PNMT

activity declines can be followed. Ideally, the investigator would perfuse the isolated adrenal of each species with artificial blood that contains various concentrations of dexamethasone or hydrocortisone and then correlate steady-state PNMT activities with glucocorticoid concentrations. However, the relatively long periods of time necessary to attain each steady-state make this sort of experiment unfeasible.

VI. SELECTIVE SECRETION OF EPINEPHRINE OR NOREPINEPHRINE FROM THE ADRENAL MEDULLA

On the basis of the evidence reviewed above, one can hypothesize that the adrenal medulla contains a single clone of chromaffin cells, which can or cannot synthesize and store epinephrine depending upon their hormonal milieu. Chromaffin cells chronically perfused with high glucocorticoid concentrations (*i.e.*, cells that receive most of their blood supply from the intramural portal circulation) will tend to have a relatively high PNMT activity and will convert a major fraction of their norepinephrine to epinephrine; cells perfused with arteriolar blood that has not received the secretions of the adrenal cortex will have low PNMT activity and will methylate relatively little norepinephrine. It should be possible to convert a "norepinephrine cell" to an "epinephrine cell" by altering its hormonal milieu, and *vice versa*. These transformations should, however, require hours or even days to take place.

If this formulation is to be accepted, it must be reconciled with a fairly large body of experimental data which indicates that appropriate stimuli can cause the adrenal gland to release selectively epinephrine or norepinephrine. It is, of course, possible that chromaffin cells near arterioles receive a separate innervation from that of cells near portal vessels; stimulation of the former nerves would cause the selective release of norepinephrine, while stimulation of the latter would liberate epinephrine. The proportion of norepinephrine to epinephrine in the adrenal venous outflow may depend upon the relative rates of blood flow to these two cell types, and this ratio, in turn, may be controlled differentially by both nerves to adrenal blood vessels and effects of ACTH on adrenocortical blood flow. Sufficient data are not available, however, even to support the hypothesis that norepinephrine cells are concentrated near arterioles and epinephrine cells near portal vessels, much less the hypotheses that norepinephrine cells have a separate innervation or separate vascular supply from epinephrine cells. Finally, it seems not at all unlikely that a given chromaffin cell can change from an "epinephrine cell" to a "norepinephrine cell," and back, on several occasions in the life of the animal; surely the cell would not be expected to change its presynaptic input or its vascular supply each time it changed.

The following discussion reviews the evidence that specific stimuli to the adrenal medulla are able to elicit the selective release of epinephrine or norepinephrine.

A. Release of catecholamines by brain stimulation

Studies performed four decades ago revealed that stimulation of certain regions of the brain could elicit catecholamine secretion from the adrenal medulla. The

effect was especially characteristic of the brainstem (33, 157, 231) and the hypothalamus (121, 166). Subsequent studies suggested that epinephrine was preferentially released upon stimulation of the hypothalamus (19, 89, 104, 205) or the amygdala (108, 206). Within the hypothalamus, however, a preferential release of norepinephrine reportedly was obtained after stimulation of the anterior portion, whereas epinephrine was released preferentially upon stimulation of areas near the mammillary bodies (205). Hence, a dual representation of the two medullary cell types within the hypothalamus was postulated (89). Prolonged stimulation (3 days) of the hypothalamus caused a 4-fold increase in adrenal tyrosine hydroxylase activity and a doubling of PNMT (207).

Electrical stimulation of the ventral cerebral cortex reportedly inhibits catecholamine secretion from the adrenals; epinephrine secretion is preferentially depressed (84).

B. Release of catecholamines by stimulation of splanchnic nerves

Stimulation of the splanchnic nerves in cats releases both epinephrine and norepinephrine from the adrenal glands (25). There is a concomitant increase in norepinephrine biosynthesis and in the methylation of this catecholamine, so that after a period of continuous stimulation the proportion of epinephrine to norepinephrine that remains in the gland actually is increased (116). This increase in adrenal epinephrine does not result from a preferential release of norepinephrine because, during stimulation, epinephrine is present in the cat adrenal venous blood in the same proportion as in the adrenal glands (116). The rise may reflect an increase in PNMT activity, perhaps secondary to an increase in glucocorticoid secretion.

Adrenomedullary secretion can be altered both quantitatively and qualitatively by varying the frequency with which the splanchnic nerves are stimulated. Catecholamine output rises with increasing frequency of stimulation, reaching a maximum (in the cat) at 30 to 60 cps (170); thereafter secretion decreases as the frequency continues to increase (169). The proportion of epinephrine in the medullary effluent also tends to increase with increasing frequency of stimulation, both in cats (71, 117, 140, 141, 172) and in dogs (179, 203); an increase in the frequency of stimulation from 2 to 20 cps raised the proportion of epinephrine in the adrenal venous effluent from 39% to 73% (203). As Mirkin (179) observed, however, in dogs this shift in the epinephrine:norepinephrine ratio occurs only at the higher, nonphysiological frequencies of stimulation. Hence, it is doubtful that the catecholamine composition of the medullary effluent is discretely regulated by the discharge frequency of the splanchnic nerves, at least in this species (179). Splanchnic stimulation produced no change in the epinephrine:norepinephrine ratio in the adrnal effluent in the cow (223) or in the dog (162, 191).

C. Release of catecholamines by acetylcholine

In 1934 Feldberg *et al.* demonstrated that both nicotinic and muscarinic receptors were involved in the stimulation of adrenal medullary secretion (87). Recent investigations on perfused adrenals of dogs and cats have confirmed the participation of both types of receptors in the stimulation of medullary secretion

(31, 65, 66, 129, 217). Stimulation of splanchnic nerves or adrenal perfusion with nicotine (66) or acetylcholine (30, 66, 176) released almost equal proportions of epinephrine and norepinephrine in cats (61%, 55%, and 58% being norepinephrine); pilocarpine or muscarine, however, released predominantly epinephrine (96% and 84% of total catecholamines) (66). On the basis of these selective effects, several investigators postulated that receptors activated by pilocarpine or muscarine were mainly associated with epinephrine cells (66, 217). Both epinephrine and norepinephrine cells were thought to have nicotinic receptors (66, 217). In contrast to these findings in cats, methacholine (a muscarinic drug) caused a preferential release of norepinephrine when compared with a nicotinic drug dimethylphenylpiperazinium iodide (DMPP) in the spinal dog (217). In other studies the norepinephrine cells of perfused cat adrenals were reported to be preferentially sensitive to nicotinic drugs (176); in atropinized dogs, acetylcholine and nicotine preferentially released epinephrine (63).

The findings described are contradictory and difficult to reconcile. Species differences could be involved: some investigators used dogs, although most used cats. Another significant variable could be the composition of the perfusing fluid; with the exception of Kayaalp and McIsaac (129), who used blood for their perfusions, all of the authors used salt solutions, usually Locke's solution adjusted to pH 7.0. The perfusion of dog adrenals with Locke's solution in this pH range has been reported to cause ultrastructural changes of the chromaffin cells as well as an increase in the catecholamine output, especially epinephrine (186).

D. Release of catecholamines during insulin-induced hypoglycemia

Cannon and Rapport (33) and Houssay and Molinelli (121) were the first to note that administration of insulin resulted in marked release of adrenal pressor amines. This effect was found to require an intact splanchnic innervation of the adrenal gland (96, 112). The loci of central neurons sensitive to hypoglycemia and the effect of this physiological input on the nature of the catecholamines secreted remain subjects of controversy.

Although it is well accepted that hypoglycemia does not act directly on adrenal medullary cells, investigators disagree on the precise location of the central neurons that are sensitive to the stimulus. Some maintain that activation of the adrenal medulla during insulin-induced hypoglycemia depends on higher centers located in the brainstem or hypothalamus (60, 70, 112, 126). Others invoke thoracic spinal centers (18, 34, 126). The existing evidence indicates that spinal centers play only a minor role, if any, in the hypoglycemic response to insulin.

A preferential secretion of epinephrine after insulin-induced hypoglycemia has been reported by some authors (27, 40, 97, 112, 114, 119). In man, a 10-fold increase in epinephrine excretion was reported after insulin administration (86, 244), whereas norepinephrine excretion decreased moderately or remained normal (86).

In normal dogs, basal epinephrine secretion was 7.5 ng $\times$ gland^{-1} $\times$ kg^{-1} $\times$ min^{-1}; epinephrine comprised 82% of the total catecholamines. Among hypophysectomized dogs, basal epinephrine secretion averaged 4 ng $\times$ gland^{-1} $\times$

$kg^{-1} \times min^{-1}$. After insulin treatment, the dogs' epinephrine secretion rose only to 16 ng $\times$ gland$^{-1} \times kg^{-1} \times min^{-1}$, *versus* 32 ng $\times$ gland$^{-1} \times kg^{-1} \times min^{-1}$ in controls (258). After ACTH treatment, epinephrine secretion was restored again to normal; later insulin treatment produced considerably higher epinephrine secretion than in control dogs.

VII. CONTROL OF PNMT ACTIVITY IN MAN

The location of the human adrenal on the theoretical curve described in figure 4 determines the extent to which epinephrine synthesis might be expected to vary with glucocorticoids. If man is like the rat in this regard (*i.e.*, if PNMT activity is very high basally and if most of the catecholamine in the normal gland is methylated), an increase in adrenocortical secretion (as in idiopathic Cushing's disease) might be expected to have little or no effect on PNMT activity or epinephrine synthesis (138). A chronic decrease in glucocorticoid secretion (*e.g.*, after hypophysectomy, in idiopathic Addison's disease, during the chronic administration of low doses of steroid hormones, in patients suffering from an isolated defect in ACTH secretion (105), or in the gland contralateral to an adrenal with a functioning cortical tumor) might be expected to depress epinephrine synthesis. Essentially no experimental data are available to aid in locating the human adrenal on the curve. Data obtained from subjects suffering from various disease states suggest, however, that human adrenals are not particularly different from those of the rat. This conclusion is supported by the observation (85, 220, 247) that 78 to 91 % of the catecholamine in the normal gland is epinephrine.

Luft and von Euler (161) examined urinary epinephrine excretion after insulin administration, before and several weeks after adult women underwent hypophysectomy for palliation of breast carcinoma. They observed that the elevation in urinary epinephrine levels after insulin administration was less than half as great after hypophysectomy as before (161). The same authors examined the effects of large doses of ACTH (50 U/day) or cortisone (200 mg/day) on basal urinary catecholamine levels and found a decrease in norepinephrine excretion but no change in epinephrine. This alteration in the ratio of norepinephrine to epinephrine could reflect increased methylation of norepinephrine in the adrenal medulla, or, more likely, a reflex decline in the physiological activity of the sympathetic nervous system (secondary to hypertension) and a consequent decrease in norepinephrine release.

Most of the extra-adrenal pheochromocytomas reported have been characterized by an inability to synthesize epinephrine (61). An occasional extra-adrenal tumor does secrete the methylated catecholamine in spite of its distance from adrenocortical tissue. Goldstein and his collaborators (98) have suggested an explanation for this paradox. They demonstrated that the PNMT activity present in a sample of pheochromocytoma tissue resembled the "frog" form of the enzyme in its thermolabile characteristics. They suggested that perhaps extra-adrenal tumors that synthesize epinephrine contain the "primitive" form of the enzyme, which is independent of steroids.

Hung and Migeon (123) described a 2-year-old boy who suffered from severe

postprandial hypoglycemia, leading on occasion to seizures. The child was found to be suffering from an unusual type of pituitary insufficiency, characterized by an isolated inability to secrete ACTH. Probably as a result of impaired adrenomedullary PNMT activity, he failed to secrete epinephrine in response to hypoglycemia. The administration of relatively large doses of cortisone corrected the child's insulin sensitivity and enabled him to secrete epinephrine when challenged with insulin (123). The authors suggested that the pathophysiological basis of this child's insulin sensitivity was related to impaired epinephrine synthesis, *i.e.*, it was analogous to the situation in hypophysectomized experimental animals. Other investigators had previously noted that children suffering from idiopathic spontaneous hypoglycemia of infancy or childhood tended to improve when treated with ACTH (17, 23, 45, 165). The transient hypoglycemia seen in newborn infants of diabetic mothers is correlated with subnormal levels of epinephrine in the urine and may reflect an "adrenal exhaustion phenomenon" (228). The ontogenesis of PNMT activity in the human adrenal apparently has not yet been examined; significant enzyme activity has been observed, however, in fetal para-aortic tissue (94).

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