Pharmacologic Tools in Autonomic Nervous System Research

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THE PERIPHERAL SYMPATHETIC NEURON is one component of a communications system which transmits information from the brain to effector organs other than skeletal muscle (*e.g.*, vascular smooth muscle, adipose cells, pineal parenchymal cells). All of the peripheral sympathetic neurons in the body are probably representatives of a single type of cell.‡ All have cell bodies in peripheral sympathetic ganglia, receive a physiologic input of acetylcholine (which is liberated presynaptically within the ganglia), and respond by releasing norepinephrine from their endings.

The fate of norepinephrine in the sympathetic neuron has been well studied. It seems safe to say that more is known about the biochemistry of this cell than about any other nerve cell in the body. This situation is certainly related to the fact that sympathetic nerve endings are present in peripheral organs as a "pure preparation"; they are not mixed with cell bodies or dendrites nor are they located among cholinergic nerve endings. Hence they are easier to study. However, credit must also be given to the imaginative application of pharmacologic tools and principles which has characterized a decade of investigation into the autonomic nerve system.

All of the enzymes involved in the synthesis of norepinephrine have been isolated, and specific inhibitors and isotopic assays have been developed for each. The loci at which norepinephrine is stored within the sympathetic nerve endings have been identified by use of anatomic and ultracentrifugation techniques. The concentration and turnover of norepinephrine in tissue have been estimated in normal subjects as well as in animals in which its fate has been perturbed by drugs or physiologic manipulations. The major metabolic transformations which can befall norepinephrine have been described, and agents have been developed which interfere specifically with each of these.

This report describes the various steps involved in the synthesis, storage, release, and inactivation of norepinephrine, as well as the effects of drugs on these processes.

Biosynthesis of Norepinephrine and Epinephrine

Only three tissues in the adult mammal produce norepinephrine. These are the sympathetic nerve endings,[1] certain parts of the brain,[2] and the chromaffin cells of the adrenal medulla.[3] In all three, the uptake of the amino acid, tyrosine, from the blood initiates synthesis (fig. 1). An active transport mechanism facilitates this uptake process, at least in the brain.[4] The tyrosine is first ring-hydroxylated to form the catechol amino acid, dihydroxyphenylalanine (dopa), through the action of an enzyme, tyrosine hydroxylase[5] (fig. 2). The tissue distribution of this enzyme is roughly parallel to that of norepinephrine and appears to be highly specific in its choice of substrates.[5] An ubiquitous[6] enzyme, aromatic L-amino acid decarboxylase, next decarboxylates dopa and forms the parent catecholamine, dihydroxyphenylethylamine (dopamine).[7] The decarboxylating enzyme is not specific; it acts also on such amino acids as 5-hydroxytryptophan, histidine and tyrosine to form serotonin,

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‡ Sympathetic cholinergic cells are not considered in this discussion.

histamine and tyramine.[8] The final step in the biosynthesis of norepinephrine involves the hydroxylation of the β-carbon of the dopamine side chain [9-11] (fig. 2). The enzyme which catalyzes this reaction (dopamine-β-oxidase) is relatively specific in its choice of substrates; besides synthesizing norepinephrine, it can also convert tyramine to octopamine.[12] Like tyrosine hydroxylase, dopamine-β-oxidase is localized to tissues that make norepinephrine.[9-11]

In the adrenal medulla much of the norepinephrine formed is subsequently N-methylated by the enzyme, phenylethanaolamine-N-methyltransferase (PNMT), to form the hormone epinephrine.[13] In adult mammals this enzyme is highly localized to the adrenal chromaffin cells.[14] Adrenocortical steroids control PNMT activity; these hormones are delivered to the medulla in high concentrations via the adrenal venous portal system.[15] Norepinephrine in tissue is present almost exclusively within sympathetic nerve endings (*e.g.*, "cardiac norepinephrine" is really the catecholamine present in the cardiac sympathetic nerves).[16] The total amount of norepinephrine in a given organ is surprisingly constant even in the presence of marked changes in the physiologic activity of its adrenergic neurons.[17] Tissue norepinephrine levels also show relatively little variability among animals of the same species. Thus it seems likely that an efficient self-regulatory mechanism exists which rapidly accommodates norepinephrine production to demand. One such mechanism might involve changes in the rate of norepinephrine synthesis. This rate could be controlled at one of two points: the conversion of tyrosine to dopa or the hydroxylation of dopamine to form norepinephrine. Since most tissues contain relatively large amounts of aromatic L-amino acid decarboxylase, it is doubtful that the decarboxylation of dopa to form dopamine can limit the synthesis of norepinephrine.[8]

Changes in the rate of tyrosine hydroxylation can lead to parallel changes in the formation of norepinephrine. When the preganglionic fibers to the superior cervical ganglia are cut, thereby decreasing the number of impulses which reach the sympathetic nerve endings in the rat salivary gland, the conversion of tyrosine, but not dopa, to norepinephrine in this

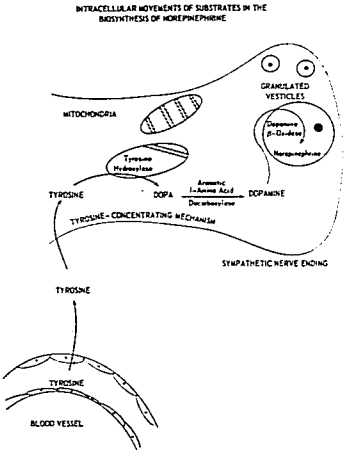

FIG. 1. Intracellular migrations in the biosynthesis of norepinephrine in the sympathetic nerve ending.[36]

organ is markedly decreased.[18] Conversely, when the cervical ganglia are stimulated electrically, the production of norepinephrine from tyrosine, but not from dopa, is increased in the salivary gland.[19] Similar effects of nerve stimulation have been observed in the rat heart following stimulation of the stellate ganglion [20] and in the guinea pig vas deferens following stimulation of the hypogastric nerve.[21, 22] These data indicate that: (1) tyrosine hydroxylation parallels norepinephrine synthesis, at least under certain experimental conditions; and (2) physiologic stimuli can control norepinephrine synthesis by an action at this enzymatic locus. These experimental procedures which alter the rate of tyrosine hydroxylation *in vivo* have not been found to produce any effect on tyrosine hydroxylase activity measured *in vitro*.[23] Hence, the precise locus of control may be at the level of the availability of substrate or cofactors to the enzyme.

When norepinephrine is added to tyrosine hydroxylase assay mixtures in a concentration of 2×10^{-4} M, the activity of the enzyme is depressed.[5] A decline in tyrosine hydroxylation can also be observed *in vivo* in animals

treated with large amounts of norepinephrine and adrenergic blocking agents.§ These data suggest that the concentration of the transmitter itself may regulate the synthesis of norepinephrine, perhaps by an action at the locus of tyrosine hydroxylation.

Recent investigations have shown that very low concentrations of norepinephrine (1.2×10^{-5} M) inhibit the conversion of dopamine to norepinephrine.[24] Moreover, stimulation of the hypogastric nerve apparently increases the rate of formation of ^{14}C-norepinephrine from ^{14}C-dopa in the vas deferens.[25] Therefore, the maintenance of tissue norepinephrine levels might also involve control of the rate of dopamine-β-oxidation.

The biosynthesis of norepinephrine can be blocked pharmacologically at several sites. Tyrosine hydroxylation is inhibited competitively by alpha-methyl para-tyrosine.[26] This agent, which lowers norepinephrine levels in experimental animals,[26] recently has been found useful in blocking the elevated production of catecholamines found in patients with pheochromocytoma.[27] It has not been possible to produce sufficient pharmacologic inhibition of aromatic L-amino acid decarboxylase activity to lower tissue norepinephrine levels. The drug alpha-methyl-dopa originally was designed to reduce norepinephrine synthesis by this mechanism; however, it was shown subsequently that its ability to lower tissue norepinephrine levels resulted from an entirely different mechanism, discussed below (the "false neurotransmitter") [28] (fig. 3). Norepinephrine synthesis may be suppressed at the final step also, by treating animals with disulfiram (Antabuse). This agent is transformed in the body to diethyl-dithiocarbamate, which chelates the copper moiety in dopamine-β-oxidase and thus reduces the activity of the enzyme.[29, 30] Patients who are treated chronically with disulfiram sometimes develop severe hypotension, which can be treated with exogenous norepinephrine. The mechanism of this hypotension probably involves an impairment in the conversion of dopamine to norepinephrine.[31]

§ Udenfriend, S. Personal communication.

Storage of Norepinephrine

All of the tissues which produce norepinephrine store the bulk of the catecholamine within specialized subcellular organelles. When

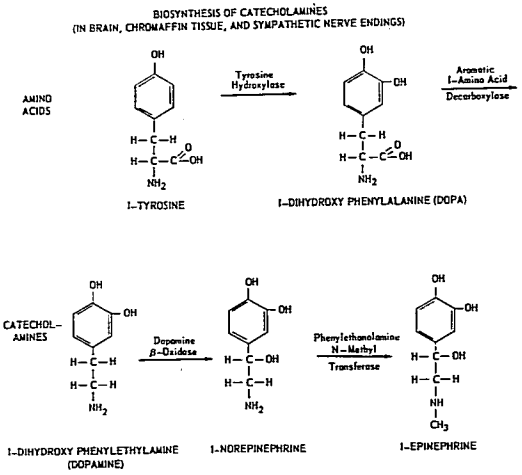

FIG. 2. Biosynthesis of catecholamines.[26]

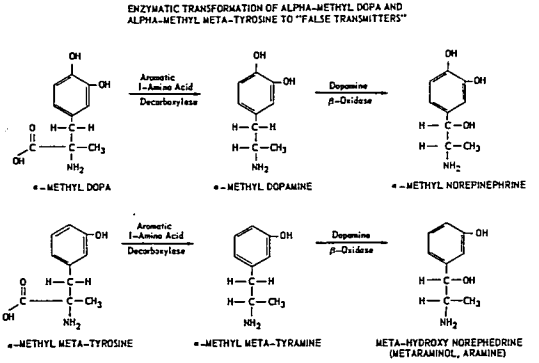

FIG. 3. *In vivo* conversion of alpha-methyl-dopa and alpha-methyl meta-tyrosine to "false neurotransmitters." [36]

viewed with the electron microscope, these structures characteristically appear as vesicles which contain a densely-staining osmophilic core.[32] The chromaffin granules of the adrenal medulla are the largest of the catecholamine storage granules. They are 500 to 4,000 Å in diameter,[33] whereas the granular synaptic vesicles in sympathetic nerve endings are approximately 400 to 500 Å.[34, 35] The catecholamine storage granules can be isolated from tissues by ultracentrifugation techniques and recognized by electron microscopy.

In addition to storing norepinephrine the granular vesicles participate in the synthesis and inactivation of the amine. They contain most or all of the dopamine-β-oxidase in the cell.[9, 10, 24] Presumbaly tyrosine is hydroxylated in a particle similar to the mitochondrion, and dopa is decarboxylated within the cytoplasm (fig. 1). The dopamine is then taken up by the granular vesicle and converted to norepinephrine, which is stored.[36] After being released from the sympathetic nerve ending, norepinephrine can be taken up again and held in the storage vesicle in a chemically unchanged but physiologically inert form.[17] This mechanism of uptake and binding, discussed below, provides a major means for the physiologic inactivation of norepinephrine. It seems likely that the protein core of the granular vesicle is synthesized in the region of the cell nucleus and migrates down the axon by the process of "axoplasmic flow." [38, 39] Apparently the granule is not able to carry out the last step in norepinephrine biosynthesis (*i.e.*, the hydroxylation of dopamine to norepinephrine) until it reaches the presynaptic region.[24, 40, 41]

The storage of norepinephrine in granular vesicles is blocked by such drugs as reserpine and segontin. Treatment of rats with 2.5 mg./kg. of reserpine results in a marked depletion of tissue norepinephrine as well as in structural changes in the granular vesicles.[33, 42] Segontin appears to be more selective in its effects on the storage of norepinephrine: within the heart, it depletes the nerve endings to the myocardium of their norepinephrine but does not affect the amine in vascular nerve endings.[43] Since the resulting pattern of norepinephrine distribution resembles that seen in the hibernating hedgehog, it was reasoned that segontin might be useful in preventing the arrhythmias which sometimes accompany clinical hypothermia. In preliminary experiments, cats treated with the drug showed considerably better survival than control animals when body temperature was lowered to 17.5° C.[43]

Certain drugs can be taken up by nerve endings and stored in the granular vesicles in place of norepinephrine. When the nerves are stimulated physiologically or pharmacologically, these agents or their metabolic products are liberated from the nerve ending as "false neurotransmitters" [44] (fig. 3). In general, the false transmitters are considerably less potent in

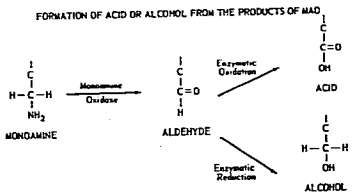

FIG. 4. Formation of acids and alcohols from monoamines by monoamine oxidase.[36]

producing receptor effects than norepinephrine itself; hence their administration results in a functional decrease in sympathetic nervous activity. There are several types of false neurotransmitters: (1) drugs which are taken up by the vesicle without prior metabolic transformation; (2) drugs which are first converted in the body to a compound acceptable to the granule; and (3) substances which are produced endogenously from food.

An example of the first type of agent is metaraminol (Aramine). Large single doses of this compound administered parenterally liberate norepinephrine from nerve endings and thereby raise blood pressure. However, smaller oral doses administered chronically are stored and released in place of the norepinephrine.[45, 46] The administration of metaraminol in this manner actually can be used in the treatment of hypertension. The second group is characterized by alpha-methyldopa (Aldomet). This compound is transformed in the body to alpha-methyl norepinephrine, taken up, and stored as a false neurotransmitter [47] (fig. 3). The release of the alpha-methyl norepinephrine in place of norepinephrine results in a clinically useful fall in blood pressure. An example of an endogenous false neurotransmitter is octopamine, which is formed within the granular vesicle by the β-hydroxylation of tyramine.[48] Under normal circumstances essentially all of the tyramine which enters the body in foodstuffs such as cheese or wine, or which is produced by the decarboxylation of tyrosine, is destroyed by oxidative deamination (fig. 4). However, when patients are treated chronically with monoamine oxidase (MAO) inhibitors, the tyramine persists and can be taken up by nerve endings and transformed into octopamine. The hypotension which sometimes occurs in patients treated chronically with MAO inhibitors may result from the release of octopamine as a false neurotransmitter.[49]

Fate of Norepinephrine

Although the level of norepinephrine in a given tissue is quite constant, individual molecules of norepinephrine turn over at a rapid rate. This turnover may be studied in several ways. Norepinephrine synthesis may be blocked (*e.g.*, with alpha-methyl para-tyrosine) and the decline in the concentration of the compound used as a measure of its rate of turnover.[50, 51] Similarly, the intraneural destruction of norepinephrine may be blocked (*e.g.*, with MAO inhibitors) and the increase in tissue norepinephrine concentration used as an indication of its rate of synthesis.[50, 51] Both of these methods, however, involve a disturbance of the normal steady-state conditions of norepinephrine metabolism.

If the regulatory mechanisms suggested above do exist, alteration in the concentration of norepinephrine may affect its rate of synthesis. Two methods now exist which permit the study of norepinephrine turnover without disturbing its normal steady-state relations. These involve the use of trace amounts of radioactively labelled tyrosine or norepinephrine. In the first case animals are given ^{3}H-tyrosine and the appearance of labelled norepinephrine is taken as a measure of rate of synthesis.[2] Tyrosine appears to be the best precursor of norepinephrine to use to study its rate of turnover since it enters all tissues following intravenous administration (dopamine does not) and is also the physiologic circulating precursor of norepinephrine (dopa is not). In the second case, animals receive ^{3}H-norepinephrine and its uptake into and subsequent disappearance from the tissues is followed.[52]

When ^{3}H-norepinephrine is administered intravenously, it is taken up by the sympathetic nerve endings and the adrenal medulla and then appears to be treated in the same manner as the endogenous compound.[53] Although very little circulating norepinephrine is taken up in the brain,[54] the norepinephrine stores in this tissue can be labelled by adminis-

tering the compound directly into the lateral cerebral ventricle.[55] The turnover of norepinephrine in the sympathetic nerve endings and the brain is rapid, while that in the adrenal medulla is slow.[2, 3, 56]

The turnover of norepinephrine is affected greatly by the physiologic state. The synthesis and release of the compound are increased following exercise or exposure to cold [57]; during hibernation (in the ground squirrel) this rate is markedly reduced.[58] The turnover of norepinephrine is elevated by experimental hypertension.[59]

The fate of all norepinephrine in tissue is not homogeneous. Some of the transmitter is being liberated from the granule into the cytoplasm continually. Most of this material is inactivated rapidly by oxidative deamination. This process, catalyzed by the enzyme MAO, results in the removal of the amine group and the formation of an aldehyde, which may be oxidized further to amino acid or reduced to an alcohol [60] (fig. 4). MAO is present within the mitochondria of adrenergic nerve endings.[61] The inhibition of this enzyme by drugs such as iproniazid causes a rise in tissue norepinephrine concentration.[62]

Some norepinephrine is not metabolized in the cytoplasm but is secreted from the cell. This occurs particularly during periods of high release which follow nerve stimulation or the administration of certain drugs which are structural analogues of the catecholamine. Such drugs are termed "sympathomimetic amines" [63]; they include amphetamine (Benzadrine), ephedrine, tyramine, and metaraminol. These compounds may act by competing with norepinephrine for intraneuronal storage sites. Some sympathomimetic amines (*i.e.*, tyramine) are metabolized by MAO in the same manner as norepinephrine. As a result their duration of action is relatively short. Amphetamine and metaraminol are protected from oxidative deamination by the presence of methyl groups on their alpha-carbon atoms; hence their actions last longer. Certain sympathomimetic drugs, such as phenylephrine (neosynephrine), are able to exert a direct effect on the postsynaptic receptor site.

The release of norepinephrine from sympathetic nerve endings can be suppressed through the action of drugs such as bretylium, guanethidine (Ismalin), and some of the MAO inhibitors.[64, 65] It is not known whether this effect of MAO inhibitors is related to their action on MAO itself.

Once norepinephrine is liberated from the nerve ending, it can undergo a variety of fates. A small but extremely important fraction of this "free" norepinephrine carries out the work of the sympathetic nerve cell by stimulating the postsynaptic loci. It is generally held that specific macromolecules exist whose stimulation is a necessary prerequisite to the physiologic actions of the catecholamines. These loci, which have not been isolated, are often termed "receptors." Several categories of receptors have been defined operationally. In general, the stimulatory effects of norepinephrine on smooth muscle contraction are said to involve α-receptors; these effects can be blocked by dibenzylene.[66] The inhibitory effects of epinephrine and isoproterenol are said to be mediated by β-receptors.[66] These actions can be blocked by dichloroisoproterenol (DCI), pronethalol (Nethalide) and propranalol. Certain physiologic actions of norepinephrine cannot be attributed to either α- or β-receptors.

Most of the free norepinephrine is inactivated rapidly by being taken up again within the sympathetic nerve ending.[67] The effect of a nerve impulse on the sympathetic nerve ending can be compared to squeezing a wet sponge: norepinephrine is allowed to leak out, but it rapidly and actively re-enters the "sponge" once the nerve impulse has passed. When this re-uptake process is blocked by cocaine or its analogues, the inactivation of free norepinephrine is markedly slowed and its physiologic effects are potentiated.[68]

Some of the free norepinephrine is destroyed in the region of the nerve ending by enzymatic inactivation: it is transformed to normetanephrine through the action of catechol-O-methyltransferase.[69, 70] Another portion of the neurotransmitter is carried away from its site of action by the circulation: it may then be O-methylated, deaminated or conjugated in the liver and kidney or may be excreted as the unchanged catecholamine (about 1 to per cent) [36] (fig. 5).

Circulating catecholamines can originate as

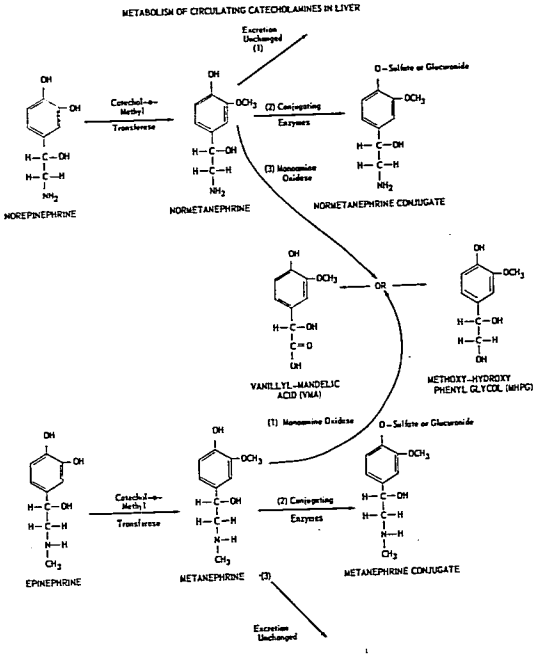

FIG. 5. Metabolism of circulating catecholamines.[36]

adrenomedullary secretion or as the overflow from sympathetic nerve stimulation. Some of the catecholamine which enters the blood stream is taken up by sympathetic nerve endings all over the body and may then be released as a neurotransmitter. As much as 20 per cent of the norepinephrine in the rat heart may originate not from synthesis in the cardiac sympathetic nerves but from uptake from the circulating blood.[71]

Most of the norepinephrine produced in the body never manages to leave the nerve ending in a physiologically active form: as described above, it is destroyed within the nerve by oxidative deamination. The major product of this reaction, dihydroxymandelic acid (DHMA), is O-methylated and excreted in the urine as vanillylmandelic acid (VMA). Hence, urinary VMA cannot be taken as a good index of sympathetic nervous activity. Measurement of this metabolite does provide a useful estimate of total catecholamine biosynthesis and thus is helpful in the diagnosis of such disease states as pheochromocytoma.[72] An estimate of sympathetic nervous tone can be obtained by measuring the free norepinephrine in the urine; similarly, adrenomedullary activity can be monitored by assaying urinary epinephrine. This approach is not entirely satisfactory, however, since an inconstant fraction of the catecholamine entering the blood stream is excreted unchanged and most of the catecholamine liberated at nerve endings is immediately taken up again *in situ*.

Understanding the Interactions of Drugs and the Sympathetic Nervous System

It is frequently possible to explain the clinical effects of a particular drug in terms of the

schema for norepinephrine metabolism described above. Hence, some drugs appear to inhibit sympathetic nervous tone by blocking the synthesis (*e.g.*, alpha-methyl para-tyrosine, disulfiram) or release (*e.g.*, guanethidine) of the neurotransmitter. Other agents (reserpine, segontin) produce a similar net effect by depleting the norepinephrine from the nerve endings or by being stored and released in place of the neurotransmitter (*e.g.*, alpha-methyldopa). Agents like cocaine exert a primary action at the locus of norepinephrine uptake; by blocking the re-entry of norepinephrine into the nerve ending, they potentiate the effects of sympathetic nerve stimulation. The sympathomimetic amines (*e.g.*, metaraminol, amphetamine) mimic the effects of nerve stimulation by releasing bound norepinephrine in a physiologically active form.

The more-or-less rational consequences of these drugs are a joy to the biochemical pharmacologist and a convenience to the clinician. It must be remembered, however, that not all of the drugs which act on the sympathetic nervous system have a single metabolic action, and that the net effect of a drug on a particular patient results from many factors, known and unknown, including his age and sex, the time of day that the drug is administered, his prior experience with this and other drugs, and his general state of health.

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Drugs

ETHACRYNIC ACID The acute hemodynamic effects of intravenous ethacrynic acid (100 mg.) were studied in 27 patients. One hour following the drug there were significant decreases in pulmonary blood volume, cardiac output and stroke volume. Urine flow in the study period ranged from 800 to 1,400 ml. The physiologic basis for the beneficial effects of this drug in the treatment of pulmonary edema is related to decreased pulmonary blood volume and previously noted decreased plasma volume. (*Samet, P., and others: Acute Effects of Intravenous Ethacrynic Acid Upon Cardiovascular Dynamics, Amer. J. Med. Sci. 255: 78 (Jan.) 1968.*)

QUININE BLINDNESS Blindness due to quinine overdose can be treated only by producing retinal vasodilation. The most effective known method for producing this is stellate ganglion block. (*Bricknell, P. P., and others: Stellate Ganglion Block in Treatment of Total Blindness Due to Quinine, Brit. Med. J. 2: 400 (Nov.) 1967.*)