THE DYNAMICS OF NOREPINEPHRINE IN THE NEURONAL CELL BODIES AND TERMINALS

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ABSTRACT

THE DYNAMICS OF NOREPINEPHRINE IN THE NEURONAL CELL BODIES AND TERMINALS

By

Ranbir Krishna Bhatnagar

Steady state concentrations of norepinephrine are maintained in peripheral tissues by synthesis and by reuptake of released norepinephrine despite variable and prolonged sympathetic stimulation. The relative importance of these two mechanisms during periods of varying activity of noradrenergic neurons is not well understood, but it appears that the properties of storage, uptake, and metabolism of norepinephrine in the terminals are different from those in the cell bodies. The purpose of the present study was to investigate the factors that regulate the content of norepinephrine in cell bodies and terminals of noradrenergic nerves during stimulation and post-stimulation periods.

The contents of norepinephrine in superior cervical ganglia, which represent cell bodies, and in submaxillary salivary glands and nictitating membranes, which contain terminals of postganglionic sympathetic neurons, were determined in cats following various treatments. The synthesis of norepinephrine was inhibited by

α-methyltyrosine infused through both common carotid arteries, and the reuptake of norepinephrine was blocked by intravenous injections of desmethylimipramine. Decentralized preganglionic fibers were stimulated unilaterally at 2 or 10 hz for 1 or 3 hours. The experiments were designed so that contralateral tissues served as the appropriate controls.

In decentralized, nonstimulated preparations, 3 hours of α-methyltyrosine infusion reduced the norepinephrine content in the cell bodies but not in the terminals. Desmethylimipramine did not alter the norepinephrine content of cell bodies or terminals.

Low or high frequencies of preganglionic stimulation, alone or in combination with desmethylimipramine or α-methyltyrosine, did not alter the norepinephrine content in the cell bodies. On the other hand, 1-3 hours of stimulation at 10 hz partially depleted norepinephrine in the terminals. α-Methyltyrosine enhanced the stimulus-induced depletion at both low and high frequencies, whereas desmethylimipramine increased the depletion of norepinephrine only at high frequencies. The α-methyltyrosine-induced depletion of norepinephrine did not result from replacement by α-methylnorepinephrine.

Preganglionic stimulation had little effect upon the conversion of tyrosine-^{14}C to norepinephrine-^{14}C in cell bodies but markedly increased the formation of

norepinephrine-^{14}C in terminals. α-Methyltyrosine reduced the synthesis of norepinephrine-^{14}C in all tissues.

To examine the dynamics of norepinephrine in cell bodies and terminals following periods of intense stimulation,the contents and rates of synthesis of this amine were determined immediately after,and at 2 and 6 hours after,the cessation of 3 hours of preganglionic stimulation. In the cell bodies, neither the endogenous norepinephrine contents nor the formation of norepinephrine-^{14}C from tyrosine-^{14}C was altered at any time. In the terminals, partial restoration of the norepinephrine content occurred within 2 hours after cessation of stimulation,but did not return to control values during the next 4 hours. Partial restoration of norepinephrine was prevented by α-methyltyrosine but not by desmethyl-imipramine. The rate of formation of norepinephrine-^{14}C was accelerated during the 30 minute period immediately after cessation of stimulation,but thereafter decreased progressively as the endogenous norepinephrine concentrations increased. At 6 hours after cessation of stimulation, when the endogenous concentrations of norepinephrine were still reduced, the rate of conversion of tyrosine-^{14}C to norepinephrine-^{14}C was not different from nonstimulated controls.

These results suggest that in cell bodies synthesis of norepinephrine proceeds at a rapid rate that is

independent of nerve activity, and concentrations of norepinephrine are maintained only by synthesis. In terminals, synthesis of norepinephrine proceeds at a slow rate in the absence of nerve activity; with low frequencies of stimulation concentrations of norepinephrine are partially maintained by synthesis,and at higher frequencies by both synthesis and reuptake. When the norepinephrine contents of terminals are extensively depleted by stimulation, synthesis only partially restores the norepinephrine concentrations;suggesting that,if the rate of norepinephrine synthesis is regulated by a feedback control mechanism,only part of the neuronal stores of norepinephrine participate in this regulation.

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INTRODUCTION

The hypothesis of chemical mediation of synaptic transmission and the role of the cell body in the homeostasis of nerve function was formulated by Scott in 1906:

> "I put forward the hypothesis that in the body of the nerve cell a substance is formed from the nucleus and Nissl bodies which gradually passes into the nerve fibres; and also that stimulation of other cells by a nerve fibre is brought about by the passage of some of this substance into the cells on which the fibre acts... The nerve cells secrete a substance the passage of which from the nerve endings is necessary to stimulation."

It is now recognized that norepinephrine functions as a chemical transmitter at terminals of the peripheral sympathetic nervous system (Euler, 1956). Much of the knowledge of the pharmacological, biochemical and physiological aspects of the noradrenergic function has been derived by altering either the affinity of post synaptic receptors for norepinephrine or the storage, metabolism, release and uptake of norepinephrine at the nerve terminals (Goodman and Gilman, 1970). Less is known about the dynamics of norepinephrine in other parts of the neuron although some effort has been made to examine the properties of this amine in the cell body (Costa *et al.*,

1961; Reinert, 1963; Norberg, 1967; Csillik *et al.*, 1967; Jacobowitz and Woodward, 1968; Van Orden *et al.*, 1970a) and the axon (Roth *et al.*, 1967; Livett *et al.*, 1969; Dahlström and Häggendal, 1970).

The disposition and dynamics of norepinephrine in cell bodies appear to be quite different from that in the terminals of noradrenergic neurons. For example, in the cell bodies norepinephrine is located in the supernatant of cell homogenates or immature vesicles, whereas in the terminals most of the norepinephrine is contained in well defined vesicles (Fischer and Snyder, 1965; Costa, 1970). The turnover of norepinephrine in ganglia (cell bodies) is much faster than in the terminals (Fischer and Snyder, 1965; Brodie *et al.*, 1966) and cocaine blocks the uptake of norepinephrine at noradrenergic nerve terminals but not at cell bodies (Fischer and Snyder, 1965). Most of the protein synthesis takes place in the cell bodies; little RNA is found in the axons and nerve terminals (Hydén, 1958). The functional significance of these differences is not fully understood nor always appreciated. For example, these differences can complicate the interpretation of the action of drugs on norepinephrine dynamics in those tissues (brain, gut, vas deferens, uterus) that contain both cell bodies and terminals (Costa, 1970). Furthermore, many efforts to demonstrate a functional role of norepinephrine in ganglionic

transmission have been contradictory and inconclusive (Costa, 1961; Reinert, 1963; Weir and McLennan, 1963; Eccles, 1964; DeGroat, 1967). The purpose of the present study was to compare the effects of neuronal activity on the dynamics of norepinephrine in cell bodies and terminals of the same neuron, and evaluate the significance of the differential characteristics in the disposition of norepinephrine at these two sites of the neuron.

Anatomical Considerations

The cell bodies of the preganglionic nerve fibers of the peripheral sympathetic nervous system are located in the central nervous system. The multipolar cell bodies of postganglionic noradrenergic neurons are generally located in autonomic ganglia (superior cervical, stellate, coeliac, inferior mesentric). In some regions, gut, vas deferens, urinary bladder, uterus, they are located intramurally. Estimates of the number of neurons in the superior cervical ganglia range from 20,000-30,000 in the cat, 63,625 in the squirrel monkey and 1,041,652 in the human to 35,528 cells/mm^3 in monkey (Ebbeson, 1968; Giacobini, 1970). Long or short unmyelinated axons (0.2-1 μ in diameter) emanate from cell bodies to innervate smooth muscle (nictitating membranes, iris, gut, uterus), cardiac tissue and glands. The axons terminate in the effector tissue as a "ground-plexus" of knobbed enlargements or varicosities (Ranson and Clark, 1959; Norberg,

1967; Dahlström and Häggendal, 1970). The distance between the axon terminal and the effector cell is 200-500 Å (Van Orden *et al.*, 1967). Some axons terminate on the cell bodies of their origin (axon collaterals) along with the terminals of the interneurons (Norberg, 1967; Jacobowitz and Woodward, 1968; Van Orden *et al.*, 1970a).

Localization and Storage

Using bioassay, chemical and histochemical techniques, sympathetic ganglia have been shown to contain high contents of norepinephrine (Vogt, 1954; Kirpekar *et al.*, 1962, Norberg, 1967). The norepinephrine content of superior cervical ganglia in dog ranges from 3-12 µg/g and that of stellate ganglia from 3.8-5.5 µg/g (Vogt, 1954). Autonomic ganglia of the cat contain 6-25 µg/g norepinephrine (Norberg and Hedqvist, 1966). When a ganglia homogenate is placed on a sucrose density-gradient and subjected to ultracentrifugation most of the norepinephrine is recovered in the supernatant fraction (Fischer and Snyder, 1965). The norepinephrine recovered in the supernatant fraction represents "soluble" or "free" norepinephrine. The advent of fluorescence microscopy supported this conclusion as norepinephrine in cell bodies was found to be diffusely distributed (Norberg, 1967). Nevertheless, in most autonomic ganglia the nerve terminals of axon collaterals or interneurons that surround the cell bodies contain characteristic dense core vesicles

which are presumably filled with norepinephrine (Norberg and Hedqvist, 1966; Phillipu *et al.*, 1967; Jacobowitz and Woodward, 1968; Van Orden *et al.*, 1970a). The vesicular proteins could be free in the axoplasm or be present in mature or immature vesicles or in mitochondria (Grafstein, 1969). Therefore, norepinephrine could be present in cell bodies both in "granular" and "agranular" forms. Nevertheless, it is not clear as to what proportion of ganglionic norepinephrine is "granular" and whether the various storage forms of norepinephrine in cell bodies represent a morphological or functional entity. The disposition of norepinephrine in ganglia is further complicated by the presence of norepinephrine-containing "chromaffin" cells (Norberg and Hedqvist, 1966; Van Orden *et al.*, 1970a).

The norepinephrine contents of postganglionic nerves which contain the axons of neuronal cell bodies range from 3.6 to 15 μg/g (Euler and Hillarp, 1956; Geffen and Rush, 1968). This norepinephrine is largely located and bound within dense core storage vesicles (Euler and Hillarp, 1956; Potter, 1966; Van Orden *et al.*, 1966).

The norepinephrine contents of tissues innervated by postganglionic nerves (spleen, heart, salivary glands) range from 1 to 4 μg/g (Euler and Hellner-Björkman, 1955; Euler and Hillarp, 1956; Iversen, 1967). The estimate for the concentration of norepinephrine in the nerve

terminals is 1000 to 3000 μg/g or greater (Dahlström and Häggendal, 1970).

Blaschko and Welch (1953) first identified granules in the adrenal medulla which could store norepinephrine. Since the relative amounts of norepinephrine in the noradrenergic terminals were many fold greater than those present in the axon or cell bodies, it was postulated that norepinephrine must be protected and stored in the nerve terminals in a manner similar to that in the adrenals. Subsequently norepinephrine containing particles were identified in the bovine splenic nerves (Euler and Hillarp, 1956) and in the nerve terminals of the tissues using density gradient centrifugation techniques (Potter, 1966), electron microscopy (Wolfe *et al.*, 1962; Kapeller and Mayor, 1967; Hökfelt, 1969) autoradiography (Wolfe *et al.*, 1962) and fluorescence microscopy (Dahlström and Häggendal, 1970; Van Orden *et al.*, 1970a). The storage vesicles are about 500 Å in diameter and osmophilic in nature; they contain norepinephrine which is complexed with adenine nucleotides, Mg ions and a specific protein (Douglas, 1968). The vesicles presumably contain dopamine-β-hydroxylase (Kaufman and Friedman, 1965) and can concentrate norepinephrine (Iversen, 1967). The association of the norepinephrine-concentrating vesicles with the nerve terminals was confirmed by the inability of the nerve terminals to concentrate

norepinephrine after denervation (Strömblad and Nickerson, 1961), chemical sympathectomy (Porter *et al.*, 1963) or immunosympathectomy (Levi-Montalcini and Angeletti, 1966).

In addition to their ability to concentrate norepinephrine, the storage vesicles can retrieve dopamine, which in turn is oxidized to norepinephrine. Thus, these vesicles prevent the oxidative deamination of both catecholamines by intraneuronal monoamine oxidase. The storage vesicles are thought to originate in cell bodies from where they are transported somatofugally (Dahlström and Häggendal, 1970). The $T_{1/2}$ of the vesicles has been estimated to be about 3 weeks (Dahlström and Häggendal, 1970).

Biosynthesis and Catabolism

The biosynthesis of norepinephrine from tyrosine is catalyzed by three enzymatic steps (Nagatsu *et al.*, 1964). This is illustrated in Figure 1.

The formation of dihydroxyphenylalanine (DOPA) is considered to be the rate limiting step in the formation of norepinephrine from tyrosine (Levitt *et al.*, 1965). This assumption is based on the following observations: The K_m of tyrosine hydroxylase is about 1-2 x 10^{-5}M and the tissue concentration of tyrosine is 5 x 10^{-5}M so that the enzyme is normally saturated; little DOPA and dopamine are present in peripheral noradrenergic tissues; the rate of norepinephrine synthesis is saturated only

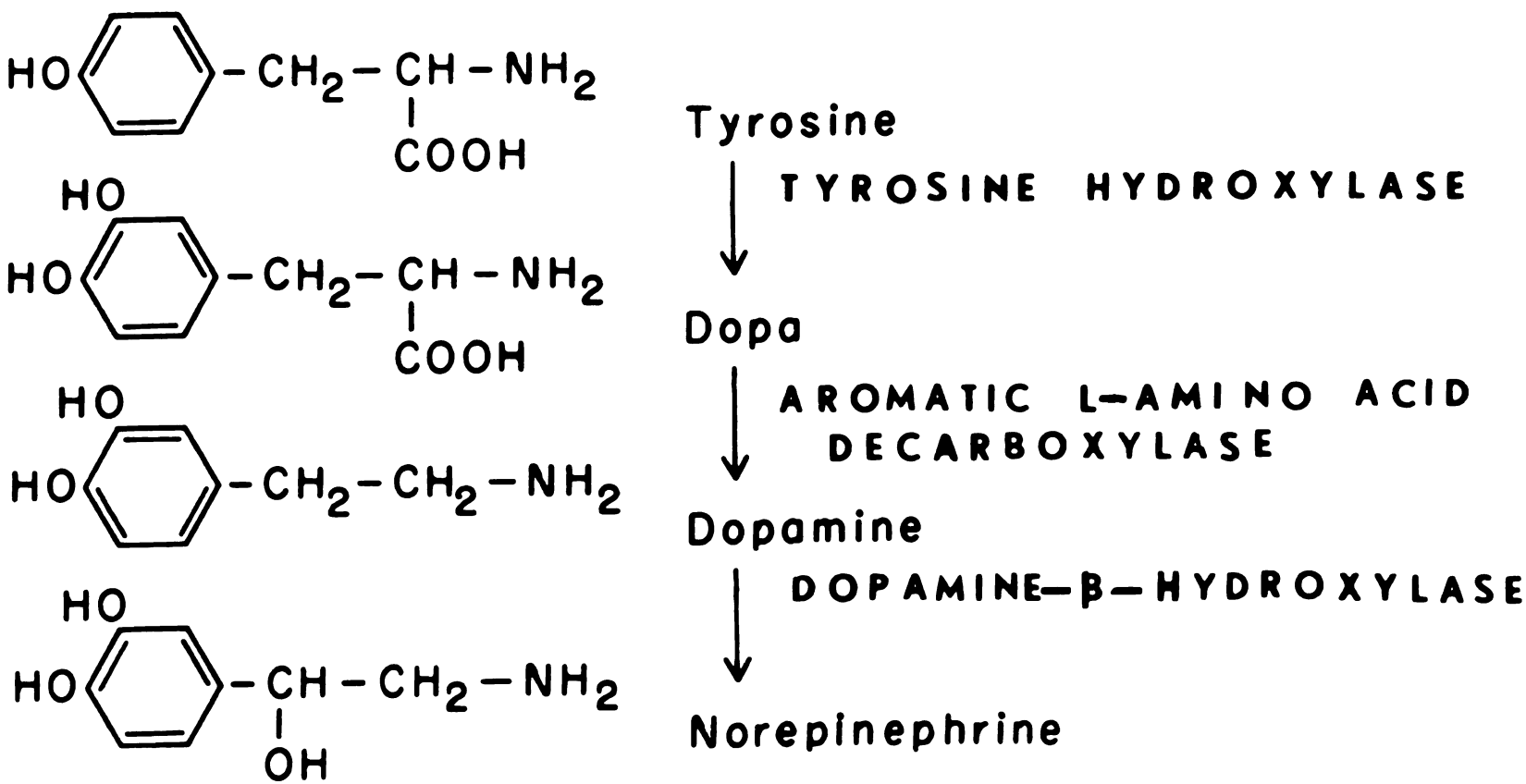

Figure 1. Pathway for the biosynthesis of norepinephrine.

with tyrosine as a precursor; rates of formation of DOPA are the same as that of norepinephrine; the activity of tissue tyrosine hydroxylase is much lower than that of DOPA decarboxylase and dopamine-β-hydroxylase; agents that markedly inhibit DOPA decarboxylase and dopamine-β-hydroxylase activity do not markedly lower tissue norepinephrine contents, whereas inhibition of tyrosine hydroxylase activity markedly lowers tissue norepinephrine contents.

Some controversy still exists over the subcellular localization of enzymes required for the synthesis of norepinephrine. Dopamine-β-hydroxylase is associated with the particulate fraction of tissue homogenates and is present in the storage vesicles in synaptosomes (Kaufman and Friedman, 1965). On the other hand tyrosine hydroxylase and DOPA decarboxylase have been identified in the cytoplasm (Laduron and Belpaire, 1968; Mussachio, 1968; Livett *et al.*, 1969; Mueller *et al.*, 1969a) and in association with "granular" vesicles (Spector *et al.*, 1967; Stjärne and Lishajko, 1967). All three enzymes are present in cell bodies (Fischer and Snyder, 1965; Mueller *et al.*, 1969a), axons (Stjärne and Lishajko, 1967; Geffen and Rush, 1968; Laduron and Belpaire, 1968; Livett *et al.*, 1969) and nerve terminals (Sedvall and Kopin, 1967a; Weiner, 1970).

Norepinephrine is enzymatically degraded by two major pathways: oxidative deamination by monoamine oxidase and methylation of the *meta* hydroxyl group by catechol-0-methyltransferase (Axelrod *et al.*, 1958; Kopin, 1964). Monoamine oxidase is present both intraneuronally and extraneuronally. Intraneuronal monoamine oxidase is located in mitochondria. Catechol-0-methyltransferase is recovered in the cytoplasmic fraction of tissue homogenates, but some may be associated with synaptic membranes (Alberici *et al.*, 1965). Both monoamine oxidase and catechol-0-methyltransferase are present in cell bodies (Fischer and Snyder, 1965; Cervoni, 1969) and nerve terminals (Kopin, 1964; Cervoni, 1969). The route of norepinephrine metabolism is dependent upon the site of metabolism. Intraneuronal norepinephrine is deaminated and extra neuronal norepinephrine is 0-methylated to form normetanephrine. Normetanephrine might be deaminated, in turn, to form 3-methoxy-4-hydroxymandelic acid. However, enzymatic degradation of norepinephrine does not seem to be the major pathway for the inactivation of physiologically released norepinephrine. Inhibition of catechol-0-methyltransferase (e.g., by pyrogallol) does not elevate the tissue contents of norepinephrine. Inhibition of both monoamine oxidase (e.g., by pheniprazine or iproniazid) and catechol-0-methyltransferase fails to potentiate noradrenergic

function (Crout, 1961). There appear to be species differences in the metabolism of norepinephrine. Monoamine oxidase inhibitors have little effect on the tissue contents of norepinephrine in the cat but increase the amine contents in the rat (Spector *et al.*, 1963).

Axonal Transport

Weiss and Hiscoe (1948) demonstrated the somatofugal transport of cytoplasmic proteins in mouse retinal ganglion cells. Similar somatofugal transport of norepinephrine and proteins has been confirmed for different species in both sensory and motor neurons and in the central nervous system (Grafstein, 1969; Dahlström and Häggendal, 1970). In sciatic and splenic nerves norepinephrine is transported somatofugally; in splenic nerves dopamine-β-hydroxylase is also transported in this manner (Geffen and Rush, 1968; Laduron and Belpaire, 1968; Livett *et al.*, 1969; Dahlström and Häggendal, 1970).

There is a slow and a fast component for axonal transport of protein (Grafstein, 1969). The slow component might serve in the maintenance and renewal of structural elements of the neuron and the fast component as a communication mechanism between cell bodies and the nerve terminals. The fast axonal transport presumably occurs through the neurotubules. Estimates for the rate of transport of the slow component vary from 0.2 to

1 mm/day and for the fast component from 100 to 930 mm/day (Grafstein, 1969). The rate of somatofugal transport of norepinephrine and newly synthesized protein is identical (5 mm/hour) in noradrenergic nerves (Kapeller and Mayor, 1967; Livett *et al.*, 1968).

The assembly of neurosecretory granules is initiated in the cell bodies. Proteins that are synthesized in cell bodies might be directly incorporated into cell organelles or vesicles for movement through the axonal matrix or be free in the axoplasm and mitochondria (Grafstein, 1969; Dahlström and Häggendal, 1970). The membranes, granules and synaptic vesicles carried by axoplasmic stream tend to settle at all terminal cul-de-sacs of axons (Lubinska, 1964).

The axonal transport of norepinephrine and proteins is independent of somatic influences and nerve impulse flow. Inhibition of protein synthesis (Peterson *et al.*, 1967) or electrical stimulation (Geffen and Rush, 1968) does not alter the rate of axonal transport of norepinephrine or proteins. Disruption of neurotubules by colchicine prevents the axonal transport of norepinephrine (Dahlström and Häggendal, 1970). Although norepinephrine in the cell bodies and axons does not appear to contribute to the minute to minute requirements of this amine at nerve terminals, the norepinephrine synthesis in the nerve terminals seems to be dependent upon a continual

supply of enzymes required for the biosynthesis of this amine (Livett *et al.*, 1968).

Release

Transmission of excitation from noradrenergic nerves to cardiac tissue, smooth muscle and glands is the result of depolarization of the effector membrane caused by norepinephrine released from the nerve terminals on arrival of the action potentials. Uptake of Ca^{++} is thought to be associated with the coupling of excitation and release of norepinephrine. An estimate of release has been obtained by measuring the responses of effector organs and by collecting and analyzing the venous effluents for norepinephrine following nerve stimulation of heart, spleen, liver and intestines. Both endogenous and exogenously administered norepinephrine are released from the nerve terminals following electrical stimulation (Blakeley *et al.*, 1964; Gillespie and Kirpekar, 1965). In those preparations where venous effluent was not collected the reduction in tissue contents of norepinephrine was considered as an indirect measure of release of norepinephrine (Fredholm and Sedvall, 1966).

Much of the knowledge about neuronal release of norepinephrine has been obtained from studies with the adrenal medulla (Douglas, 1968) and with nerve terminals in the spleen (Brown and Gillespie, 1957). Little is known about the release of norepinephrine from the ganglia.

Small amounts of norepinephrine were released in the venous effluent of isolated perfused superior cervical ganglia following preganglionic nerve stimulation (Bülbring, 1944). Norepinephrine was detected in the venous outflow from intact and chronically decentralized ganglia (Reinert, 1963). Orthodromic and antidromic stimulation of ganglia increased the norepinephrine content in the venous outflow but this increase was not consistently obtained (Reinert, 1963). Electrical stimulation of isolated splenic nerves did not cause the release of norepinephrine (Roth *et al.*, 1967).

Release of small amounts of norepinephrine occurs spontaneously from the nerve terminals as indicated by the excitatory junction potentials. Spontaneously released norepinephrine presumably occurs in "quantal packets" (Burnstock and Holman, 1966). The basal rate of norepinephrine release could be increased many fold on stimulation of noradrenergic nerves supplying the tissues; the stimulus-induced release is frequency dependent (Brown, 1960; Folkow *et al.*, 1967). At low frequencies of stimulation (0.5-2 hz) less norepinephrine is released than at higher frequencies (5-10 hz) (Haefely *et al.*, 1965; Folkow *et al.*, 1967).

Stimulus-induced release of norepinephrine in isolated perfused spleen at 0.5 hz is 1.5 ng/stimulus and at 1-2 hz, 4.5 to 5.5 ng/stimulus. With higher

stimulation rates (4-8 hz) no further increase is found in the release of norepinephrine (Haefely *et al.*, 1965). At low frequencies of stimulation the output of norepinephrine from the nerve terminals could be sustained during prolonged periods of stimulation (Euler and Hellner-Björkman, 1955; Folkow *et al.*, 1967). With frequencies higher than 10 hz norepinephrine overflows into circulation, responses of effector tissue reach a maximum, and the tissue is partially depleted of norepinephrine. Therefore, it is thought that physiological frequency of afferent input is around 2 hz with 8-10 hz being the upper limit of tonic sympathetic discharge (Brown, 1960; Folkow *et al.*, 1967).

In adrenal glands both norepinephrine storage vesicles and dopamine-β-hydroxylase are intimately associated with ATP and Mg ions. The contents of the storage vesicles are extruded by exocytosis (Douglas, 1968; Viveros *et al.*, 1969). Evidence for the release of storage vesicles from noradrenergic terminals is controversial (Stjärne *et al.*, 1970) although stimulus-induced release of synaptic vesicles and dopamine-β-hydroxylase from spleen has been reported (Geffen *et al.*, 1969; Gewirtz and Kopin, 1970).

Disposition of Released Norepinephrine

Studies of the disposition of released norepinephrine have been conducted with sympathetically innervated tissues (spleen, heart, salivary glands and intestines) (Iversen, 1967). Little is known about the fate of norepinephrine which is supposedly released from the cell bodies. The norepinephrine released at the nerve terminals can be disposed in at least three ways: reuptake into the nerve terminals, diffusion into the circulation, enzymatic degradation.

Sympathetically innervated tissues can concentrate circulating norepinephrine (Strömblad and Nickerson, 1961; Whitby *et al.*, 1961); the rate of accumulation depends on the blood flow and density of noradrenergic innervation (Kopin *et al.*, 1965). The uptake of norepinephrine into the nerve terminals is an active process (Trendelenburg, 1969). Norepinephrine in the synpatic cleft is transported across the neuronal membrane and then into the storage vesicles. The transport of norepinephrine at the neuronal membrane is blocked by cocaine, imipramine, desmethylimipramine and phenoxybenzamine (Brown, 1960; Dengler *et al.*, 1961; Axelrod *et al.*, 1962; Sigg *et al.*, 1963; Fischer and Snyder, 1965). Desmethylimipramine and reserpine also block the transport of intraneuronal norepinephrine into the storage vesicles (Brodie *et al.*, 1968). Blockade of

uptake of norepinephrine into the nerve terminals results in an elevation in the norepinephrine contents in the perfusate of isolated spleen following stimulation of splenic nerves (Brown, 1960), a decrease in the uptake of norepinephrine and norepinephrine-^{3}H by a variety of tissues (Axelrod *et al.*, 1961) and an increase in the contraction of nictitating membranes following electrical stimulation of sympathetic nerves (Sigg *et al.*, 1963). Surgical denervation (Strömblad and Nickerson, 1961) or chemical sympathectomy (Jonsson and Sachs, 1970) greatly reduce the uptake of exogenously administered norepinephrine. The results of the above studies indicate that most of the norepinephrine released at nerve terminals is reincorporated into the nerve terminals. This mechanism is believed to be the major means for inactivating neuronally released norepinephrine (Axelrod *et al.*, 1961). Norepinephrine which is retrieved into the nerve terminals is also available for release (Blakeley *et al.*, 1964).

The fate of norepinephrine released from ganglia is unknown but the ganglia can retrieve circulating norepinephrine (Fischer and Snyder, 1965). However, cocaine does not block the uptake of norepinephrine into the ganglia (Fischer and Snyder, 1965).

Some of the norepinephrine that is released into the synaptic cleft diffuses into the blood. The amount

of diffusion is also frequency dependent (Brown, 1960). Within the physiological range of stimulation frequencies little norepinephrine diffuses ("overflows") into the circulation (Brown, 1960; Haefely *et al.*, 1965; Folkow *et al.*, 1967). At "supraphysiological" frequencies (>12-14 hz) or after the blockade of reuptake of norepinephrine considerable amounts of norepinephrine overflow into the circulation. Reuptake mechanisms do not compensate for the excess release of norepinephrine at higher frequencies indicating that this process is saturable (Folkow *et al.*, 1967). Approximately 70-80% of released norepinephrine is reincorporated into the terminals.

The balance between release and uptake of norepinephrine can be sustained indefinitely if the norepinephrine released per minute is below 1-1.5% of total tissue norepinephrine contents (Folkow *et al.*, 1967; Iversen, 1967). The possibility remains, however, that during depolarization uptake of norepinephrine is reduced (Palaic and Panisset, 1969; Folkow *et al.*, 1967), and therefore at high frequencies of stimulation less norepinephrine might be reincorporated into the nerve terminals.

The disposition of released norepinephrine by enzymatic degradation is negligible (Kopin, 1964; Jonason, 1969). Monoamine oxidase is present both intraneuronally and extraneuronally. Perhaps some norepinephrine that is

reincorporated into the neuron is deaminated by intra-neuronal monoamine oxidase before it is transported into and stored in the vesicles (Kopin, 1964). Part of the norepinephrine is 0-methylated in presence of catechol-0-methyltransferase. Inhibition of monoamine oxidase and catechol-0-methyltransferase, however, does not potentiate the effector tissue (e.g., heart) responses to norepinephrine (Crout, 1961).

Turnover

Most of the studies of noradrenergic function during the last decade have focused on the alteration of norepinephrine contents of tissues. It has become apparent now that turnover or renewal of norepinephrine is of major importance in the study of noradrenergic function because turnover and not the norepinephrine contents of tissue signifies the functional status of the neuron (Costa, 1970). The turnover rate implies that a steady state exists such that the synthesis and transport of norepinephrine into a metabolic pool equals its catabolism and release (Neff *et al.*, 1969). Various methods have been used to determine the turnover rate of norepinephrine in both neuronal cell bodies and terminals by measuring: a) the rate of decline of exogenously administered norepinephrine-^{3}H of high specific activity (Montanari *et al.*, 1963; Fischer and Snyder, 1965); b) the rate of decline of neuronal norepinephrine contents after

inhibition of the biosynthesis of this amine by α-methyl tyrosine (Spector *et al.*, 1965; Brodie *et al.*, 1966); c) the rate of decline in specific activity of norepinephrine after administration of labelled precursors of norepinephrine (Burack and Draskóczy, 1964); d) the rate of recovery of neuronal norepinephrine after its depletion (Spector *et al.*, 1962); e) the rate of increase of neuronal norepinephrine contents after inhibition of monoamine oxidase (Costa, 1969); f) the rate of conversion of tyrosine-^{14}C to norepinephrine-^{14}C (Neff *et al.*, 1969).

The turnover of norepinephrine appears to be dependent on neuronal activity and on the neuronal population of tissues; it varies in different parts of the neuron. The estimate of the rates of synthesis of norepinephrine in cell bodies (Brodie *et al.*, 1966; Costa, 1969), axons (Roth *et al.*, 1967) and noradrenergic terminals are 1.53, 3.0 and 0.12 to 0.22 μg/g/hr respectively. The $T_{1/2}$ of norepinephrine in peripheral noradrenergic neuroeffector organs is approximately 12-18 hours whereas the $T_{1/2}$ of ganglionic norepinephrine is about 2 hours (Fischer and Snyder, 1965; Brodie *et al.*, 1966; Costa, 1969). Decentralization slows the disappearance of norepinephrine-^{3}H from ganglia; decentralization for two weeks also results in an elevated ganglionic content of norepinephrine (Kirpekar *et al.*, 1962; Fischer and Snyder, 1965). The

turnover of norepinephrine in the neuronal terminals of heart, salivary glands and spleen is also reduced after chronic decentralization.

Regulation of Neuronal Content of Norepinephrine

Despite variable and prolonged sympathetic stimulation, steady state concentrations of norepinephrine remain constant at a level that is characteristic for each individual tissue. Stimulation results in the neuronal release of norepinephrine but does not cause a significant change in the concentration of norepinephrine in the cell bodies (Reinert, 1963) or nerve terminals (Luco and Goñi, 1948; Euler and Hellner-Björkman, 1955; Folkow *et al.*, 1967). Decentralization slows the disappearance of norepinephrine in neuronal cell bodies and terminals and with time elevates the norepinephrine concentration of soma (Kirpekar *et al.*, 1962; Fischer and Snyder, 1965). These results suggest that changes in sympathetic activity influence the neuronal contents of norepinephrine by altering the rate of synthesis of norepinephrine. The regulation of synthesis of neuronal norepinephrine has been studied after acute or chronic alterations in sympathetic nerve activity following stress, exercise, exposure to cold, α-receptor blockade, electrical stimulation of noradrenergic nerves, thyroidectomy, denervation and hypertension (Dairman and Udenfriend, 1970; Weiner, 1970). Three

major mechanisms have been suggested for the regulation of neuronal contents of norepinephrine: (1) rapid changes in the norepinephrine content resulting from the removal of tyrosine hydroxylase from product inhibition, (2) gradual changes resulting from alterations in the amount of tyrosine hydroxylase and (3) reuptake of norepinephrine from the synaptic cleft.

Electrical stimulation has been frequently used to alter the sympathetic discharge in order to study the noradrenergic mechanism. Stimulation of the stellate ganglion *in vivo* results in an increased formation of norepinephrine in the rat heart from labelled tyrosine but not from labelled DOPA (Gordon *et al.*, 1966). During the *in vivo* stimulation of decentralized preganglionic fibers the rat submaxillary gland accumulates 5-times more norepinephrine-^{14}C than the contralateral decentralized nonstimulated gland following the administration of tyrosine-^{14}C but not after DOPA-^{14}C (Sedvall and Kopin, 1967). *In vitro* stimulation of hypogastric nerves results in an increased conversion of tyrosine-^{3}H to norepinephrine-^{3}H in the guinea pig vas deferens (Alousi and Weiner, 1966; Roth *et al.*, 1966). Acute increase in noradrenergic activity resulting from α-receptor blockade (by phenoxybenzamine) or cold exposure elevates the synthesis of norepinephrine in the rat heart and adrenal glands (Dairman and Udenfriend,

1970). Since the conversion of DOPA to norepinephrine was not accelerated the results of the above studies support the concept that tyrosine hydroxylase is the rate limiting step in the biosynthesis of norepinephrine (Levitt *et al.*, 1965) and that the stimulus-induced increase in the synthesis of this amine is the result of increased tyrosine hydroxylase activity. The mechanism of increased synthesis, however, is not well understood (Gordon *et al.*, 1966).

Several attempts were made to explain the mechanism of increase in the synthesis of norepinephrine following the increase in afferent input. The increase in the norepinephrine synthesis did not appear to be due to the effector tissue activity (Roth *et al.*, 1966) or an increase in the amount of tyrosine hydroxylase (Sedvall and Kopin, 1967). Cycloheximide, an inhibitor of protein synthesis, did not block the increased synthesis of norepinephrine following exercise (Gordon *et al.*, 1966). Electrical stimulation did not alter the axonal transport of protein (Peterson *et al.*, 1967) or of norepinephrine (Roth *et al.*, 1967). An increase in the transport of tyrosine to the site where it could be more readily hydroxylated or an increase in cofactors, however, could not be excluded (Weiner and Rabadjija, 1968). Since the catechols, including norepinephrine, inhibit the activity of purified tyrosine hydroxylase it

was proposed (Nagatsu *et al.*, 1964) and later demonstrated (Weiner and Rabadjija, 1968) that norepinephrine exerts end-product inhibition of the initial and rate-limiting step in the biosynthesis of norepinephrine, that is, the hydroxylation of tyrosine. Norepinephrine presumably competes with pteridine co-factors to inhibit tyrosine hydroxylase (Nagatsu *et al.*, 1964). However, contrary hypotheses have been proposed. Considering that norepinephrine is stored and protected in the synaptic vesicles which do not contain tyrosine hydroxylase this amine could not exert inhibition of tyrosine hydroxylase (Costa, 1970); therefore, DOPA and dopamine might regulate the tissue norepinephrine contents by feedback inhibition of tyrosine hydroxylase. DOPA and dopamine are readily formed in the cytoplasm and tyrosine hydroxylase would be expected to be more accessible to these catechols than to norepinephrine (Costa, 1970). Costa's proposal does not include the possibility that small amounts of intraneuronal "free" norepinephrine could inhibit synthesis (Weiner and Rabadjija, 1968). Indeed, all norepinephrine which is retrieved into the nerve terminals from synaptic cleft is not incorporated into the synaptic vesicles, and part of it is free to be deaminated by monoamine oxidase (Kopin, 1964). Conceivably this norepinephrine could exert end-product inhibition of tyrosine hydroxylase.

The acute augmentation of sympathetic discharge did not alter the amount of tyrosine hydroxylase in the nerve terminals (Sedvall and Kopin, 1967a; Dairman and Udenfriend, 1970). Most studies on the influence of nerve activity on the neuronal contents of tyrosine hydroxylase have been conducted after the augmentation of sympathetic discharge; little is known about the effects of diminished neuronal activity. Decentralization slows the turnover of norepinephrine in the nerve terminals (Fischer and Snyder, 1965), but it does not alter the tyrosine hydroxylase activity in the adrenal glands (Thoenen *et al.*, 1969). However, in spontaneously hypertensive rats, in which there is a reflex reduction in the sympathetic activity, the amount of tyrosine hydroxylase is decreased in the blood vessels (Tarver and Spector, 1970). Again most studies on the interrelation of nerve activity and norepinephrine contents were conducted in tissues containing either the nerve terminals (e.g., salivary glands) or a mixture of cell bodies and terminals of peripheral noradrenergic neurons (e.g., vas deferens).

There is no systematic study on the regulation of norepinephrine contents in cell bodies. A few studies indicate that acute changes in neuronal activity do not alter either the activity of tyrosine hydroxylase or the norepinephrine content in ganglia (Reinert, 1963; Sedvall

and Kopin, 1967a; Dairman and Udenfriend, 1970). Tyrosine hydroxylase activity in ganglia is many fold higher than in the salivary glands (Sedvall and Kopin, 1967a). Synthesis of norepinephrine in the cell bodies may be operating at a maximal rate irrespective of the degree of neuronal activity. Indeed, electrical stimulation of splenic nerves, where the rate of synthesis of norepinephrine is 15-20 times greater than in the vas deferens, did not alter the synthesis of norepinephrine (Roth *et al.*, 1967). Since the protein synthesis in cell bodies adapts to functional demands (Hydén, 1958) the lack of change in neuronal contents of tyrosine hydroxylase was puzzling.

Recent investigations indicate that prolonged increase in afferent input increases the synthesis of norepinephrine by mechanisms other than the removal of inhibition of tyrosine hydroxylase. The accelerated synthesis of norepinephrine that is obtained in the vas deferens during electrical stimulation of hypogastric nerve could be blocked by exogenous norepinephrine but not by puromycin, an inhibitor of protein synthesis. The accelerated synthesis of norepinephrine, however, continued after the cessation of stimulation of hypogastric nerves despite little change in the norepinephrine contents of tissue. This post-stimulation increase in the synthesis of norepinephrine was blocked by puromycin

but not by norepinephrine. It was postulated that stimulation caused an increase in the amount of tyrosine hydroxylase in the vas deferens (Weiner and Rabadjija, 1968). These investigators did not measure the amount of the enzyme, but it appears that acute changes in neuronal activity do not cause an increase in the amount of tyrosine hydroxylase (Dairman and Udenfriend, 1970). Prolonged periods of drug-induced increases in sympathetic discharge (with reserpine and 6-hydroxydopamine) increase the amount of tyrosine hydroxylase in the rat superior cervical ganglia and adrenal glands (Mueller *et al.*, 1969a). This increase in tyrosine hydroxylase is blocked by cycloheximide and actinomycin-D (Mueller *et al.*, 1969b) or by sectioning the splanchnic nerves and preganglionic sympathetic fibers (Thoenen *et al.*, 1969). Chronic increases in reflex sympathetic discharge caused by repeated injections of phenoxybenzamine also increase the conversion of tyrosine-^{14}C to norepinephrine-^{14}C in the nerve terminals of heart. Part of the increase in the formation of norepinephrine-^{14}C could be attributed to an increase in the amount of tyrosine hydroxylase. Thus, a prolonged increase in the afferent input might result in a compensatory induction of the rate limiting enzyme in the biosynthesis of norepinephrine in both neuronal cell bodies and terminals in order to maintain transmitter requirements.

It would appear that the induction of tyrosine hydroxylase is initiated in the cell bodies. The labelled soluble proteins associated with the synaptic vesicles appear first in the cell bodies and then in the nerve terminals (Barondes, 1968). The neuronal increase in the amount of tyrosine hydroxylase that is obtained after drug-induced augmentation of sympathetic discharge appears two days sooner in the cell bodies than in the nerve terimals (Axelrod *et al.*, 1970). After the depletion of neuronal norepinephrine contents with reserpine norepinephrine fluorescence appears first in the cell bodies and then in the nerve endings (Dahlström and Häggendal, 1970).

Of the two major mechanisms for the regulation of neuronal norepinephrine contents, end-product inhibition and inductive changes in enzyme contents, it would appear that immediate functional demands of the neurotransmitter are met and regulated primarily by the mechanism of removal of product inhibition of tyrosine hydroxylase. Prolonged increases in neuronal activity, however, might result in inductive changes in the biosynthesis of norepinephrine. The inductive changes are gradual. It takes 12 hours for the increase in the amount of tyrosine hydroxylase to become detected after a chronic augmentation in sympathetic discharge (Mueller *et al.*, 1969a, b). On the other hand the increase in norepinephrine

synthesis from tyrosine as a result of the stimulus-induced release of this amine, and hence, removal of end-product inhibition of tyrosine hydroxylase, is rapid. The importance of the mechanism of end-product inhibition in the regulation of biosynthesis of norepinephrine is further evidenced by the following observations: $T_{1/2}$ of tyrosine hydroxylase (Mueller *et al.*, 1969a, b) and that of norepinephrine storage vesicles (Dahlström and Häggendal, 1970) are estimated to be 8 and 22 days respectively; electrical stimulation and inhibition of protein synthesis does not alter the axonal transport of protein (Peterson *et al.*, 1967) and the rate of axonal transport of enzymes and norepinephrine storage vesicles (Livett *et al.*, 1968; Dahlström and Häggendal, 1970) is not fast enough to fulfill the immediate transmitter requirements at the terminals. Furthermore, in those tissues where turnover of norepinephrine is slow (heart and salivary glands) following depletion by drugs (Spector *et al.*, 1962) or electrical stimulation (Fredholm and Sedvall, 1966) the amine contents return to control levels rapidly. It appears there is no correlation between the reduction of neuronal contents of norepinephrine and the elevation of tyrosine hydroxylase (Mueller *et al.*, 1969a). Accordingly, the terminals can independently maintain their ability to synthesize norepinephrine for a relatively long period of time.

Among the factors that regulate the neuronal contents of norepinephrine the reuptake mechanism is important, particularly at the nerve terminals. A series of events take place in noradrenergic nerve terminals upon the arrival of action potentials: norepinephrine is released, much of the released norepinephrine is retrieved and norepinephrine synthesis is accelerated. It appears that steady state levels of norepinephrine in the nerve terminals are maintained by local synthesis and by reuptake. The relative importance of these two processes in resting and active neurons, however, is still controversial (Malmfors, 1969; Hedqvist and Stjärne, 1969). For example, in nerve terminals, synthesis of norepinephrine is believed to be of prime importance at low frequencies of stimulation while reuptake of this amine predominates at higher frequencies (Bhagat and Friedman, 1969).

Clearly, the localization and the processes of storage, release and uptake of norepinephrine differ in the cell bodies and terminals of noradrenergic neurons. It appears that mechanisms for synthesis and storage of neuronal norepinephrine are initiated in the cell body and acquire different characteristics along the axon and within the nerve terminals. Although there is a distinct requirement of intact innervation for normal turnover of neuronal norepinephrine the mechanism of

regulation of norepinephrine synthesis differs in the cell bodies and terminals. Furthermore, there is a lack of systematic studies of the effect of neuronal activity on the disposition of norepinephrine in neuronal cell bodies. The differences in the properties and disposition of norepinephrine in the neuronal cell bodies and terminals is not always appreciated. Tissues that are rich in cell bodies (e.g., vas deferens, brain, gut) should not be viewed as a system of identical functional units operating in parallel with those tissues (e.g., heart, spleen, salivary glands) that are rich in nerve terminals (Costa, 1970).

In the present studies some attempt was made to understand the factors that control the dynamics of norepinephrine in both cell bodies and terminals of noradrenergic neurons during and after transient augmentation of nerve impulses. The cell bodies were represented by the superior cervical ganglia and the terminals by the submaxillary salivary glands and the smooth muscle of the nictitating membranes of cat. It will be demonstrated that in the cell bodies only synthesis contributes to the norepinephrine content; synthesis proceeds at a rapid rate and is not altered by acute electrical stimulation and for up to 6 hours after the cessation of stimulation. In the terminals, during stimulation both synthesis and reuptake of norepinephrine increase. The synthesis is

increased by removal of end-product inhibition; but the norepinephrine concentrations are not exclusively regulated by this mechanism, since the synthesis rate of norepinephrine returns to control values at a time when the tissue norepinephrine content is still reduced.

METHODS

A. Surgical and Experimental Methods

Experiments were performed on cats of either sex weighing 1.6-3.5 kg. They were anesthetized with an intraperitoneal injection of Dial Urethane (sodium diallylbarbiturate, 70 mg/kg; urethane, 280 mg/kg; monoethylurea, 280 mg/kg). The surgical preparation was essentially the same as described by Volle (1962). The trachea was exposed and intubated at the level of the clavicles. The rest of the trachea, larynx and oesophagus were either retracted through the oral cavity or sectioned at the level of mandibles. The cervical sympathetic trunks were dissected free from the vagi and common carotid arteries. Unless mentioned otherwise, the preganglionic fibers of both sides were sectioned 2-3 cms caudal to the superior cervical ganglia and the cranial end was tied with thin thread to assist with placement on electrodes. Skin flaps of neck were tied to a metal frame to form a cervical well which was filled with mineral oil. The final surgical preparation is diagrammatically illustrated in Figure 2.

Figure 2. Schematic drawing of the surgical preparation.

Preganglionic (Pre) sympathetic fibers of both sides were decentralized and stimulated unilaterally. In some experiments postganglionic (Post) fibers were stimulated. α-Methyltyrosine or 0.9% sodium chloride solution and labelled compounds were infused through both common carotid arteries which supply the blood to the superior cervical ganglia (SCG), submaxillary salivary glands (SG) and nictitating membranes (NM). Arrows indicate that the NM were connected to strain gauges with thread to record the stimulation-induced contractile responses on the polygraph.

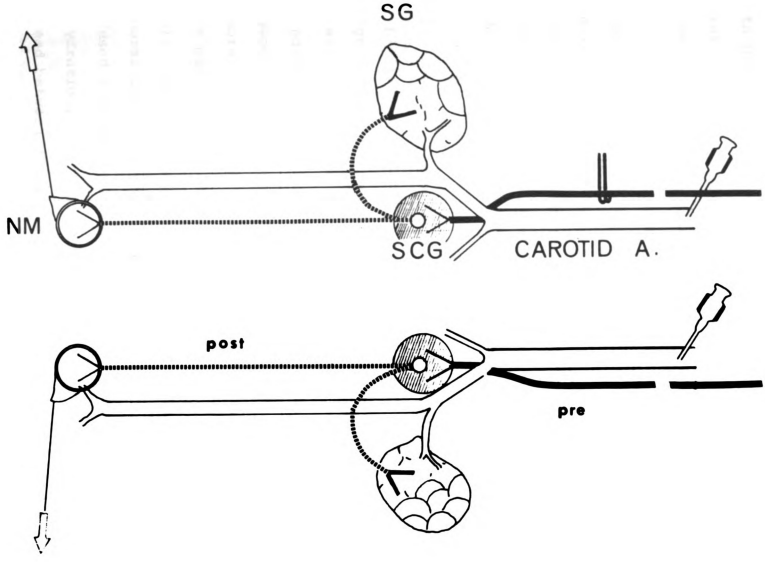

Figure 2. Schematic drawing of the surgical preparation.

Supramaximal (15 v) unilateral electrical stimulation (0.2 msec duration) of preganglionic fibers was effected with platinum electrodes using a square wave generator (Grass Model S4). The stimulation schedule was 2 hz or 10 hz for 30 seconds of each minute (intermittent) for one or three hours. In a few experiments stimulation was conducted at various frequencies for 60 seconds of each minute (continuous) for 1 to 3 hours. Effectiveness of ganglionic transmission was constantly monitored by recording nictitating membrane contractions on Grass Model 7 polygraph using a strain gauge. The initial tension on both nictitating membranes was set at 7-7.5 gms. The contralateral preganglionic fibers were placed on platinum electrodes without being stimulated. A 27 gauge needle was inserted into each common carotid artery for infusion of drugs. The needle was connected to polyethylene tubing and fitted to a holder which was clamped to the frame. Drugs were also infused into the femoral vein. Both arterial and intravenous infusions were made using a compact Harvard infusion pump (Model 975). Blood pressure was constantly monitored from a femoral artery. Sodium chloride 0.9% solution was infused i.a. or i.v. at the rate of 12 ml/hour to prevent dehydration. The body temperature of the cat was maintained between 37-38°C using an electric lamp. At the end of each experiment the ganglia, nictitating membranes

(smooth muscle, connective tissue and all orbital attachments), and salivary glands were dissected out and frozen until analyzed for norepinephrine. In experiments where radioactive isotopes were used the chest was opened and the cat was perfused with one liter of cold 0.9% sodium chloride through a cannula inserted into the aorta before the tissues were dissected out; the right auricle was excised for drainage of blood.

B. Biochemical Methods

1. Assay of α-methyltyrosine in plasma

Plasma α-methyltyrosine concentrations were determined fluorometrically according to the method of Porter *et al.* (1966) as modified by Carr and Moore (1968). Aliquots (0.1 or 0.2 ml) of plasma and known concentrations of α-methyltyrosine (6 µg and 2 µg) were mixed with 1 ml of 6% trichloroacetic acid in plastic centrifuge tubes. Samples were centrifuged at 10,000 x g for 5 min. The supernatant was transfered to 15 ml glass centrifuge tubes containing 0.1 ml of pyridine and 0.5 ml of 1% ninhydrin. The tubes were heated in boiling water bath for 10 min and cooled immediately thereafter. One-tenth ml of concentrated HCl was added to each tube and contents were mixed thoroughly. Two ml of ethylacetate were added to each tube. The tubes were stoppered and shaken for 2 min. The organic layer was discarded by aspiration. One ml of the remaining solution

was transfered to 15 ml glass centrifuge tubes and the following reagents were added: 0.5 ml of nitric acid reagent (1 ml 2.5% $NaNo_2$ + 49 ml HNO_3 (1:5 dilution of concentrated HNO_3) and 0.5 ml of 0.1% nitroso-napthol in 95% ethanol. The mixture was heated for 30 min at 55°C and allowed to cool to room temperature. Ethylene dichloride (2.5 ml) was then added, tubes were shaken for 5 min and centrifuged. The fluorescence of the supernatant was determined in an Aminco-Bowman spectrophotofluorometer at activation-fluorescent wave lengths of 456-560 mμ. Reagent blanks were determined from samples that contained 0.2 ml water instead of plasma. Tissue blanks were determined from blood that was removed before the administration of α-methyltyrosine. The unknown concentrations of α-methyltyrosine were determined from known standards.

2. Assay of endogenous norepinephrine content of superior cervical ganglia

The ganglia were cleaned on an ice-cooled Petri dish under 3x magnifying lens, weighed, and homogenized in 3 ml cold 0.4 N perchloric acid using an all glass homogenizer. The homogenizers were washed with an additional 2 ml 0.4 N perchloric acid. Washings and tissue homogenate were combined, allowed to stand for 15 min and then centrifuged at 27,000 x g for 15 min. The supernatant was transfered to beakers containing

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1 ml of 0.2 M EDTA and approximately 200 mg of aluminum oxide (Woelm). Alumina was washed according to the procedure described by Moore and Rech (1967). The alumina-tissue extracts were adjusted to pH 8.6 with 2 N, 0.2 N and 0.02 N sodium hydroxide under constant stirring with a motor driven glass rod. After the pH had stabilized at 8.6 the stirring was continued for at least five additional minutes to ensure complete adsorption of norepinephrine on alumina. The supernatant was decanted and the alumina transferred to 15 ml conical glass centrifuge tubes containing 10 ml of distilled water. The tubes were shaken for 2 min and centrifuged at 900 x g for 1 min. Water was then aspirated and the alumina washed again three more times with 5 ml portions of distilled water. After the last wash 3 ml 0.05 N perchloric acid was added to each centrifuge tube. Tubes were shaken for 15 min and centrifuged for one minute. Two 5 ml samples of 0.4 N perchloric acid containing known concentrations of norepinephrine (.05 µg and .10 µg) were treated with alumina exactly as described above for tissue extracts. Two ml aliquots of 0.05 N perchloric acid eluates from tissue and standard samples were transferred to test tubes and the remainder of the eluates from tissue samples were pooled for determination of blanks. Similarly, the remaining eluates from standard samples were pooled separately for determination of their

blanks. The fluorescent product of norepinephrine was developed as follows. To one 2 ml aliquot were added, 1 ml of 0.5 M potassium phosphate buffer, pH 7.0, and 0.05 ml of 0.25% potassium ferricyanide. Two min after the addition of potassium ferricyanide, 0.3 ml of freshly prepared alkaline ascorbate was added (1 ml of 2% ascorbic acid + 9 ml of 5 N NaOH). After each addition the mixture was agitated on Vortex Genie Mixer. Fluorescence was determined 10 min later in an Aminco-Bowman spectrophotofluorometer at activation-fluorescent wave lengths of 390-510 mμ. Blanks were determined by adding to the 2 ml aliquots of pooled alumina eluates from tissue and standard samples 1.0 ml 0.5 M potassium phosphate buffer, pH 7.0, and 0.3 ml alkaline ascorbate. Fifteen minutes later 0.06 ml potassium ferricyanide was added. The recovery of the norepinephrine standards was 73.5 ± 1% (mean ± 1 S.E., n = 25). The unknown norepinephrine concentrations were determined from these standards.

3. Assay of endogenous norepinephrine in salivary glands and nictitating membranes

The salivary glands were cleaned on an ice-cooled Petri dish, weighed, frozen inastainless steel mortar with liquid nitrogen and pulverized with a pre-cooled stainless steel pestle. The pulverized tissues were homogenized in 8.0 ml 0.4 N perchloric acid using all

glass homogenizers. The homogenizers were washed with an additional 4 ml 0.4 N perchloric acid. The tissue homogenate and washings were pooled, kept on ice for 30 min, centrifuged at 27,000 x g for 15 min, and the supernatant frozen until analyzed for norepinephrine.

The nictitating membranes were carefully trimmed, cut into small pieces, and kept overnight in 10 ml 0.4 N perchloric acid at 4°C. The suspension of tissue in perchloric acid was centrifuged at 27,000 x g for 15 min, and the supernatant was filtered through glass wool. The glass wool was washed with 2.0 ml of 0.4 N perchloric acid. The combined supernatant-wash was then analyzed for norepinephrine.

The perchloric acid extracts from the salivary glands and nictitating membranes were analyzed for norepinephrine content as described by Moore and Rech (1967); recovery of norepinephrine standard was 75 ± 2% (mean ± 1 S.E., n = 34). The norepinephrine concentrations of the unknown samples were calculated from these standards.

4. Assay of norepinephrine-^{14}C in superior cervical ganglia

The ganglia were homogenized in 2 ml of 0.4 N perchloric acid and the homogenizers were washed with 1 ml of 0.4 N perchloric acid. The tissue homogenate and washings were combined, allowed to stand for 15 min,

and centrifuged at 27,000 x g for 15 min. A 100 µl aliquot was withdrawn from the extract for the determination of total radioactivity. One µg of norepinephrine standard and 0.1 ml of 2% freshly prepared ascorbic acid was added to each sample. Two standards of norepinephrine (1.0 µg) containing known amounts of norepinephrine-^{14}C and 0.1 ml of 2% ascorbic acid were analyzed concurrently. The procedure for alumina extraction was similar to that described above for estimation of endogenous norepinephrine content with the exception that elution from alumina was performed with 3.0 ml of 0.2 N acetic acid instead of perchloric acid. The pH of the alumina eluates was adjusted to 6.0 using 2 N, 0.2 N and 0.02 N sodium hydroxide under constant stirring. Samples were then applied to columns of Dowex 50W-X4 (Na^+, 28 mm^2 x 40 mm) buffered with 0.1 M sodium phosphate buffer, pH 6.5, containing 0.1% EDTA. The columns were washed with 5 ml of distilled water. The first 5 ml of 1 N HCl eluate was discarded. Norepinephrine-^{14}C was eluted with additional 12 ml 1.0 N HCl. The eluates were collected in vials and dried under a stream of air. The residue was dissolved in 1.0 ml 0.1 N HCl. Ten ml of modified Bray's solution (6 g of 2,5-diphenyloxazole and 100 g of naphthalene/liter of dioxane) was added to each vial and radioactivity determined in a Beckman Model 100 Spectrometer with 87% efficiency. Recovery of the

norepinephrine-^{14}C standard was 53 ± 2% (mean ± 1 S.E., n = 27). No correction was made for this recovery.

5. Assay of norepinephrine-^{14}C in salivary glands and nictitating membranes

The procedure followed for alumina extraction of norepinephrine-^{14}C was similar to the one described above for the determination of total norepinephrine in these tissues. Two norepinephrine standards (1.0 µg) with known amounts of radioactivity of norepinephrine-^{14}C were analyzed concurrently. No carrier norepinephrine was added to tissue samples. The procedure for separation of norepinephrine-^{14}C on Dowex columns was similar to that described for ganglia with the exception that ascorbic acid was not added to the alumina eluates as it interfered with the determination of the total norepinephrine content. Out of 12 ml of the 1 N HCl eulates from Dowex columns, 3 ml were withdrawn for the estimation of the endogenous norepinephrine content, and 9 ml dried in scintillation vials under a stream of air. The pH of a 2 ml aliquot of 1 N HCl eluates was adjusted to 6.5-6.8 with saturated, 1 M and 0.5 M K_2CO_3, and 0.4 ml of potassium phosphate buffer, pH 6.5, was added. The fluorescent product was formed according to the procedure described above for these tissues. Recovery of norepinephrine standard was 55 ± 2% (mean ± 1 S.E., n = 18) and of norepinephrine-^{14}C 53 ± 2% (mean ±

1 S.E., n = 27). Norepinephrine content of tissues was calculated from these standards.

6. Assay of radioactive α-methylnorepinephrine-^{3}H

α-Methyltyrosine was checked for purity by ascending co-chromatography with authentic α-methyltyrosine on cellulose coated thin layer plates using butanol, glacial acetic acid, water (5:1:4) as the developing solvent; only one major peak was detected. This is illustrated in Figure 3. This peak coincided with that of authentic α-methyltyrosine and represented more than 99% of total radioactivity recovered from the plate; the remaining 1% represented an unidentified peak. The conversion of α-methyltyrosine-^{3}H to α-methylnorepinephrine-^{3}H was determined in all tissues according to the procedure outlined above for measurement of norepinephrine-^{14}C. It was found that α-methylnorepinephrine has the same elution pattern on the Dowex column as norepinephrine. α-Methylnorepinephrine in the eluates from Dowex columns was analyzed according to the procedure described above for norepinephrine with the exception that 2 ml aliquots were heated in a boiling water bath for 50 min before assay for norepinephrine. Heating increases the fluorescence intensity of α-methylnorepinephrine (Dominic and Moore, 1971).

Figure 3. Determination of the purity of α-methyltyrosine-^{3}H (αMT).

Five μg of authentic αMT and approximately 0.5 μc of αMT-^{3}H were co-chromatographed on cellulose coated thin layer plates using butanol, glacial acetic acid, water (5:1:4) as the developing solvent. Sequential one cm strips of thin layer plates were cut and αMT eluted by shaking with 1 ml of 0.1 N HCl. The radioactivity and fluorescence of αMT were determined from 0.1 ml aliquots of the eluate. αMT-^{3}H and authentic αMT had identical Rf values (0.51).

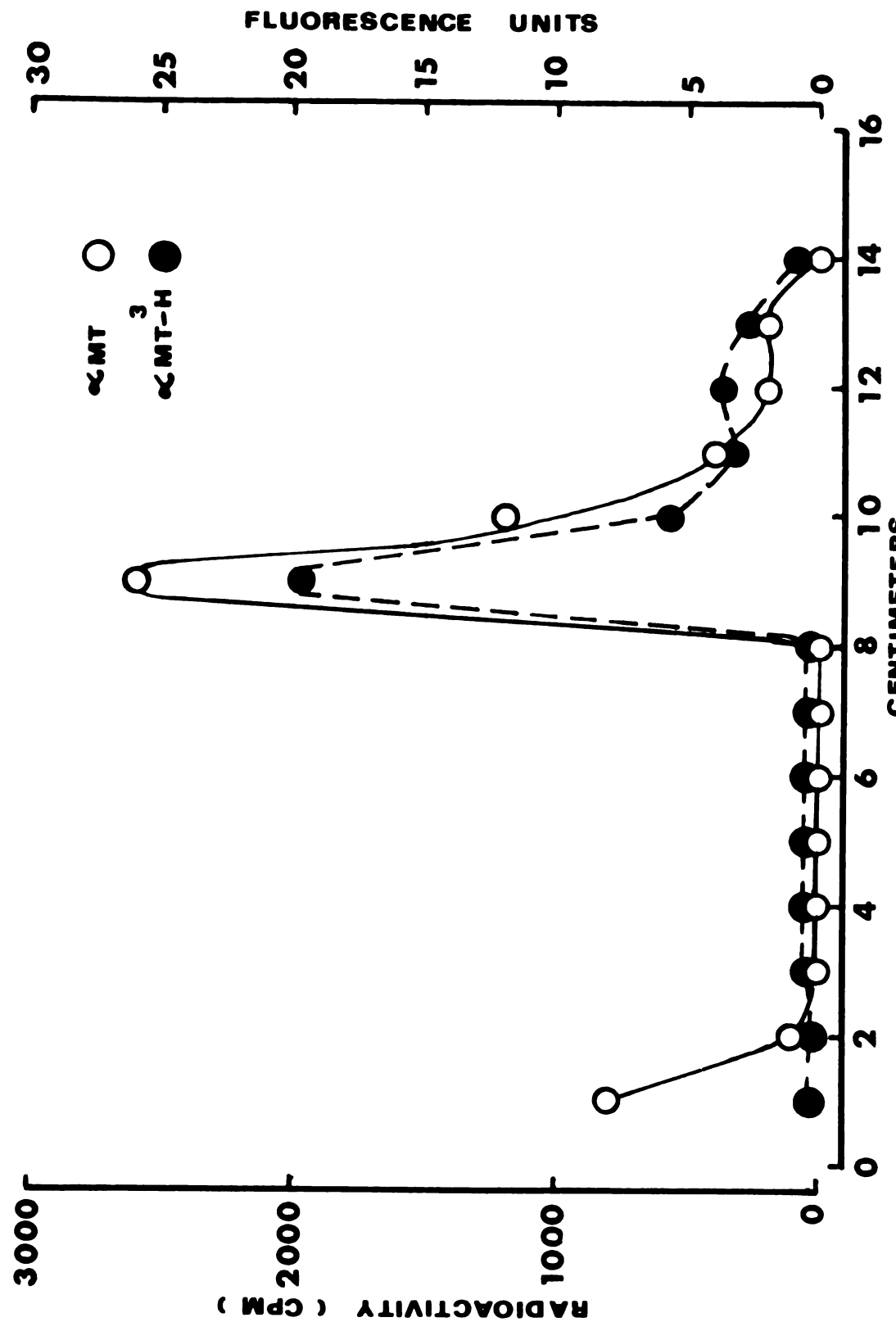

Figure 3. Determination of the purity of α-methyltyrosine-^{3}H (αMT).

7. Calculation of radioactivity in tissues

In all those experiments where isotopes were used radioactivity in the nictitating membranes is expressed as dpm per membrane whereas radioactivity in the ganglia and salivary glands is reported on the basis of wet weight. The weights chosen, 10 mg for superior cervical ganglia and 1 g for salivary glands, approximate the actual weights of these tissues (ganglia--10.9 ± 0.35 mg; n = 36; salivary glands--1.32 ± 0.06 g; n = 17). Because of variation in total radioactivity the amount of norepinephrine-^{14}C formed is expressed as an absolute amount and as a percent of total radioactivity.

C. Administration of Drugs and Isotopes

Desmethylimipramine hydrochloride (supplied by Dr. R. C. Ursillo of Lakeside Laboratories, Milwaukee, Wis.), cycloheximide (Sigma Chemical Company) and pheniprazine (Catron; JB 516; Lakeside Laboratories) were injected i.v., and L-α-methyltyrosine (supplied by Dr. C. A. Stone of Merck Institute for Therapeutic Research, West Point, Pa.) was infused through each common carotid artery at the rate of 0.1 mg/min. All drugs were dissolved in 0.9% sodium chloride; α-methyltyrosine required heating under constant stirring to dissolve. L-tyrosine-^{14}C (uniformly labelled, New England Nuclear Corporation; specific activity, 395 mc/mmol) was either infused through each common carotid artery or through the

femoral vein. L-α-methyltyrosine-^{3}H (supplied by Dr. C. Rosenblum of Merck, Sharp and Dohme, Rahway, N. J.) (specific activity 5.2 mc/mmol) was infused through each common carotid artery. 3,4-diphdroxyphenylethylamine-1-^{14}C·HBr (dopamine) (New England Nuclear Corporation; specific activity, 6.28 mc/mmol) was infused through each common carotid artery. All isotopes were checked for purity by ascending co-chromatography with authentic compounds on cellulose coated thin layer plates.

D. Statistical Methods

Statistical analysis was carried out with Student's t test (Goldstein, 1964). In most of the experiments a paired comparison was made.

RESULTS

A. The Dynamics of Norepinephrine in the Neuronal Cell Bodies and Terminals During the Resting State.

1. Norepinephrine concentrations in tissues from the right and left side of the same cat

As summarized in Table 1, there was less variation in the tissue concentrations of norepinephrine between the right and left sides of the same cat than there was between individual cats. For example, the norepinephrine content in ganglia and nictitating membranes varied from 6.78 to 13.96 μg/g and 0.18 to 1.60 μg/membrane respectively, but there was good correspondence between the right and left sides of the same cat. The same pattern was seen with the salivary glands; the norepinephrine content ranged from 1.27 μg/g to 2.40 μg/g with mean values of 1.74 ± 0.34 μg/g for the right side and 1.70 ± 0.20 μg/g for the left side. Because of the close correspondence of norepinephrine concentrations in tissues from the left and right side of the same cat, most experiments were designed so that the contralateral tissues served as appropriate controls.

Table 1. Norepinephrine concentration of tissues from right and left side of individual untreated cats.

	Superior cervical ganglia (µg/g)		Salivary glands (µg/g)		Nictitating membranes (µg/membrane)	
	Right	Left	Right	Left	Right	Left
	7.35	7.25	-----	----	0.88	0.64
	6.78	7.48	-----	----	0.92	0.85
	13.41	13.96	-----	----	0.45	0.45
	8.64	8.18	-----	----	0.26	0.18
	8.36	7.39	-----	----	0.60	0.82
	8.38	8.14	1.54	1.56	0.92	1.15
	7.45	8.51	1.27	1.43	0.92	0.89
	11.08	9.39	2.40	2.10	1.50	1.60
Mean± 1 S.E.	8.99± .77	8.70± .78	1.74± .34	1.70± .20	0.81± .13	0.82± .15

2. Plasma and tissue concentrations of α-methyltyrosine

Plasma contents of α-methyltyrosine at various times after the start of the infusion of this drug into both common carotid arteries are illustrated in Figure 4. The α-methyltyrosine content in plasma from the femoral vein approached equilibrium fairly rapidly and, as would be expected, was lower than that observed in jugular vein plasma. At 1, 3 and 5 1/2 hours the α-methyltyrosine concentrations in plasma from the right and left jugular veins were the same. If the jugular vein content of α-methyltyrosine represents the concentrations of this drug in tissues of the head regions, it could be predicted that the synthesis of norepinephrine would be inhibited by at least 70% within one hour (Udenfriend *et al.*, 1966). The tissue concentrations of α-methyltyrosine were in fact higher than those in the plasma. For example, at 1 hour the α-methyltyrosine content in the salivary glands was 174 ± 31 μg/g (mean ± 1 S.E., N of 5). This concentration is in excess of that calculated to inhibit norepinephrine synthesis by 90% (Udenfriend *et al.*, 1966). That the administration of α-methyltyrosine did indeed block the synthesis of norepinephrine is illustrated by the exponential depletion of this amine in the superior cervical ganglia (Figure 5), and by blockade of the conversion of tyrosine-^{14}C to norepinephrine-^{14}C in ganglia, nictitating membranes, and salivary glands (Table 10).

Figure 4. Plasma concentrations of α-methyltyrosine (αMT) following i.a. infusion of the drug.

αMT was infused into each common carotid artery at a rate of 0.1 mg/min for 1-5 1/2 hours. Blood samples were withdrawn from the femoral vein (●) and from the right (Δ) and left (▲) jugular veins at various times after the start of the infusion. Each point represents the mean concentration of αMT as determined from 3 to 18 experiments and the vertical lines represent ± 1 S.E.

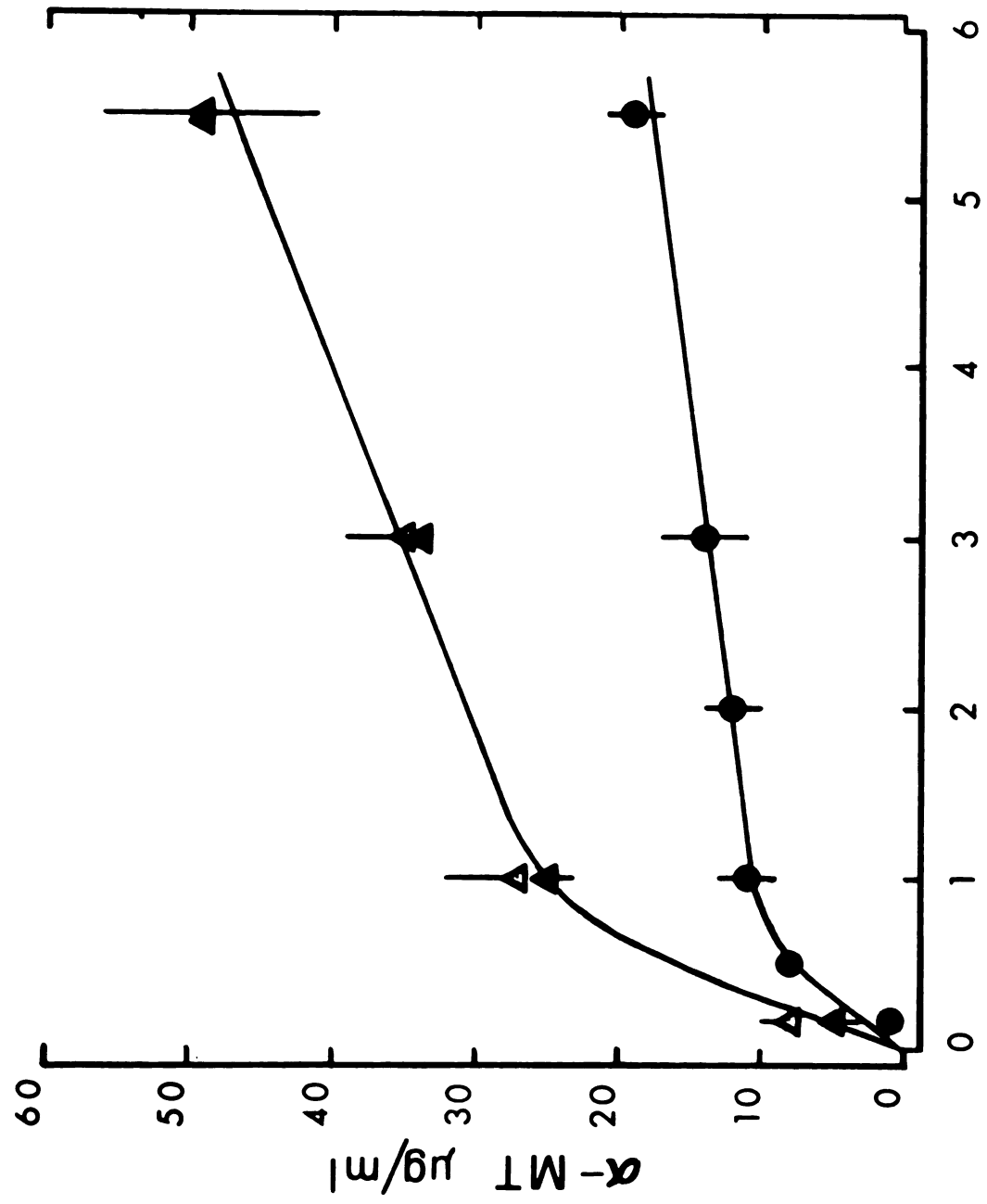

Figure 4. Plasma concentrations of α-methyltyrosine (αMT) following i.a. infusion of the drug.

Figure 5. The content of norepinephrine (NE) in nictitating membranes and superior cervical ganglia after the infusion of α-methyltyrosine (αMT).

αMT was infused at a rate of 0.1 mg/min into both common carotid arteries for 1, 3 or 5 1/2 hours. The nictitating membrane and ganglion were removed from one side, and the preganglionic fibers were sectioned on the other side just before the infusion of αMT was begun. The NE concentrations are plotted as the percentage of control; 100% represents the NE content in the superior cervical ganglia (10.4 ± 1.0 μg/g) and nictitating membranes (0.95 ± .18 μg/membrane) that were removed before the start of the αMT infusion. Each point represents the results obtained from 3 to 5 cats and the vertical lines represent ± 1 S.E.

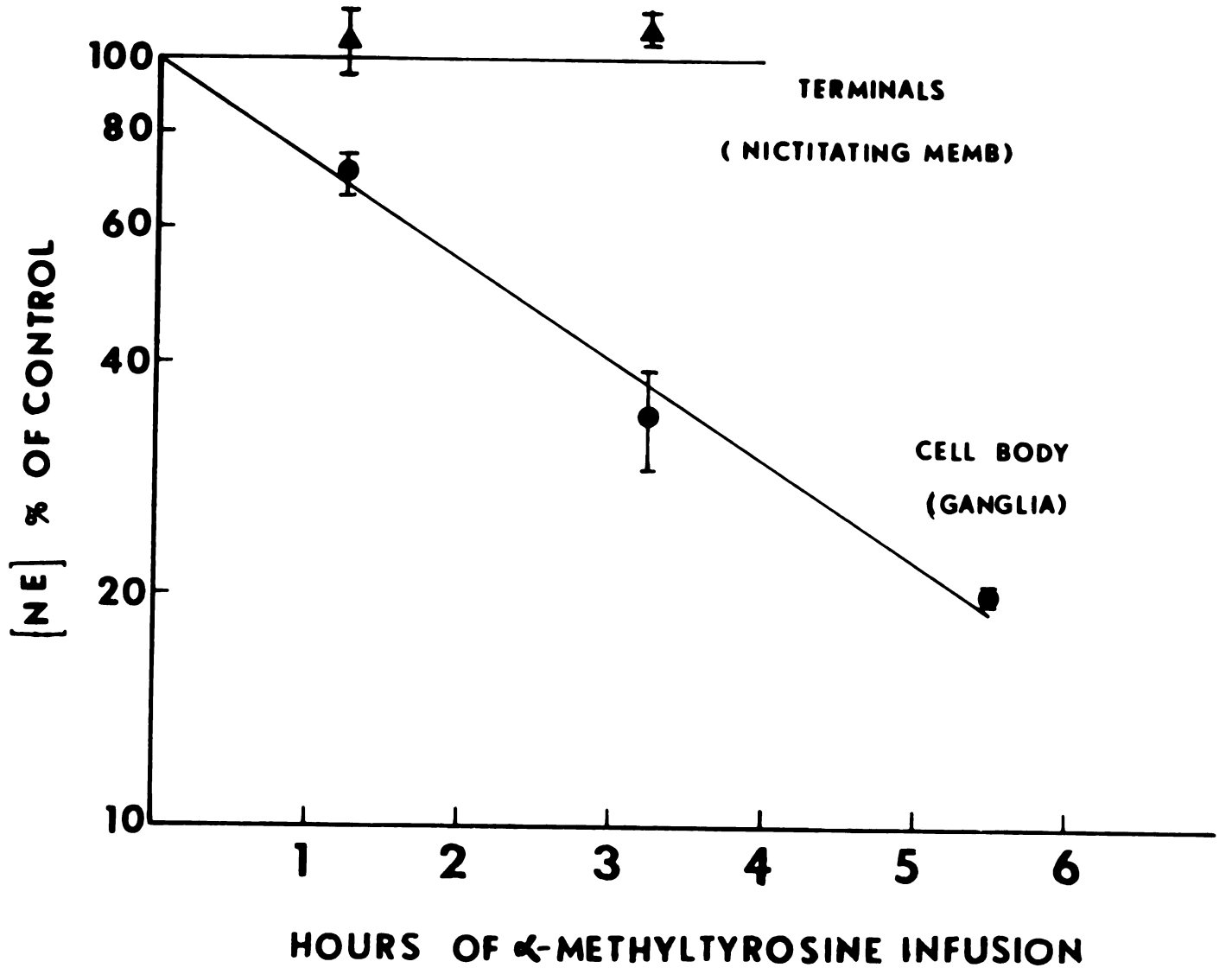

Figure 5 The content of norepinephrine (NE) in nictitating membranes and superior cervical ganglia after the infusion of α-methyltyrosine (αMT).

3. Tissue norepinephrine concentration after infusion of α-methyltyrosine

The effects of α-methyltyrosine on the norepinephrine content of nonstimulated decentralized nictitating membranes and ganglia are depicted in Figure 5. The norepinephrine content in the ganglia declined in an exponential fashion with a $T_{1/2}$ of 2 1/2 hours, whereas three hours of α-methyltyrosine infusion did not significantly alter the norepinephrine content in the nictitating membranes. These data confirm previous reports that the rate of turnover of norepinephrine is faster in cell bodies than in terminals (Brodie *et al.*, 1966). Similar results were obtained in intact or nondecentralized preparations; that is, three hours of α-methyltyrosine infusion significantly reduced the norepinephrine levels in ganglia but not in salivary glands or nictitating membranes (Table 2). Therefore, it appears that in the anesthetized cat resting neuronal activity does not markedly alter the rate of turnover of norepinephrine in either the cell bodies or terminals since the effects of α-methyltyrosine on norepinephrine contents were the same in intact and decentralized tissues.

Table 2. Tissue norepinephrine (NE) after three hours of α-methyltyrosine (αMT) infusion.

	Nondecentralized (4)			Decentralized (8)		
Tissue	Control	αMT	% of control	Control	αMT	% of control
Ganglia (μg/g)	6.74± .41	2.31± .23	35± 4 [a]	10.43± 1.02	3.70± .54	34± 5 [a]
Nictitating membrane (μg/membrane)	0.87± .13	0.85± .16	96± 5	0.93± .18	1.00± .18	110± 6
Salivary glands (μg/g)	0.96± .20	0.89± .18	93± 5	----	----	----

Control values represent the NE content of tissues that were removed before the start of the αMT infusion. All values represent the mean content of NE ± 1 S.E. as determined in 4 to 8 separate experiments.

[a] indicates those percentages that are significantly different from 100 ($P<.01$).

4. Effects of desmethylimipramine and α-methyltyrosine on the contents of norepinephrine in nonstimulated tissues

Three hours after the i.v. injection or infusion of desmethylimipramine to nonstimulated preparations, the norepinephrine content was not altered in the superior cervical ganglia, nictitating membranes, or in the salivary glands (Figure 6 and Table 3). In addition, desmethylimipramine did not alter the effects of α-methyltyrosine; that is, desmethylimipramine plus α-methyltyrosine produced the same effect as α-methyltyrosine alone. For example, three hours of α-methyltyrosine infusion alone reduced the ganglionic norepinephrine contents to 35% of control (Table 2 and Figure 6) and in presence of low (2 mg/kg) or high (10 mg/kg) doses of desmethylimipramine, the ganglionic norepinephrine content was 31 and 25% of control respectively (Table 3). These data indicate that in nonstimulated preparations norepinephrine is synthesized at a rapid rate in cell bodies but not in terminals; furthermore, in a resting neuron reuptake does not play a major role in maintaining norepinephrine concentrations in cell bodies or in terminals.

Figure 6. Tissue contents of norepinephrine (NE) following administration of desmethyl-imipramine (DMI) and/or α-methyltyrosine (αMT) in nonstimulated preparations.

The experiments were carried out in cats in which the preganglionic fibers were sectioned but not stimulated. αMT was infused through both common carotid arteries at a rate of 0.1 mg/min for three hours. DMI (2 mg/kg) was injected intravenously over a 5 minute period concomitant with the start of the αMT infusion; another injection of DMI (1 mg/kg) was made two hours later. The height of each bar represents the mean content of NE expressed as a percentage of the NE content in the tissues that were removed prior to the administration of αMT and DMI. Vertical lines projected on each bar represent 1 S.E. as determined from 3 experiments. Asterisks mark those values that are significantly different from 100% ($P<.01$). SCG - superior cervical ganglia; NM - nictitating membranes.

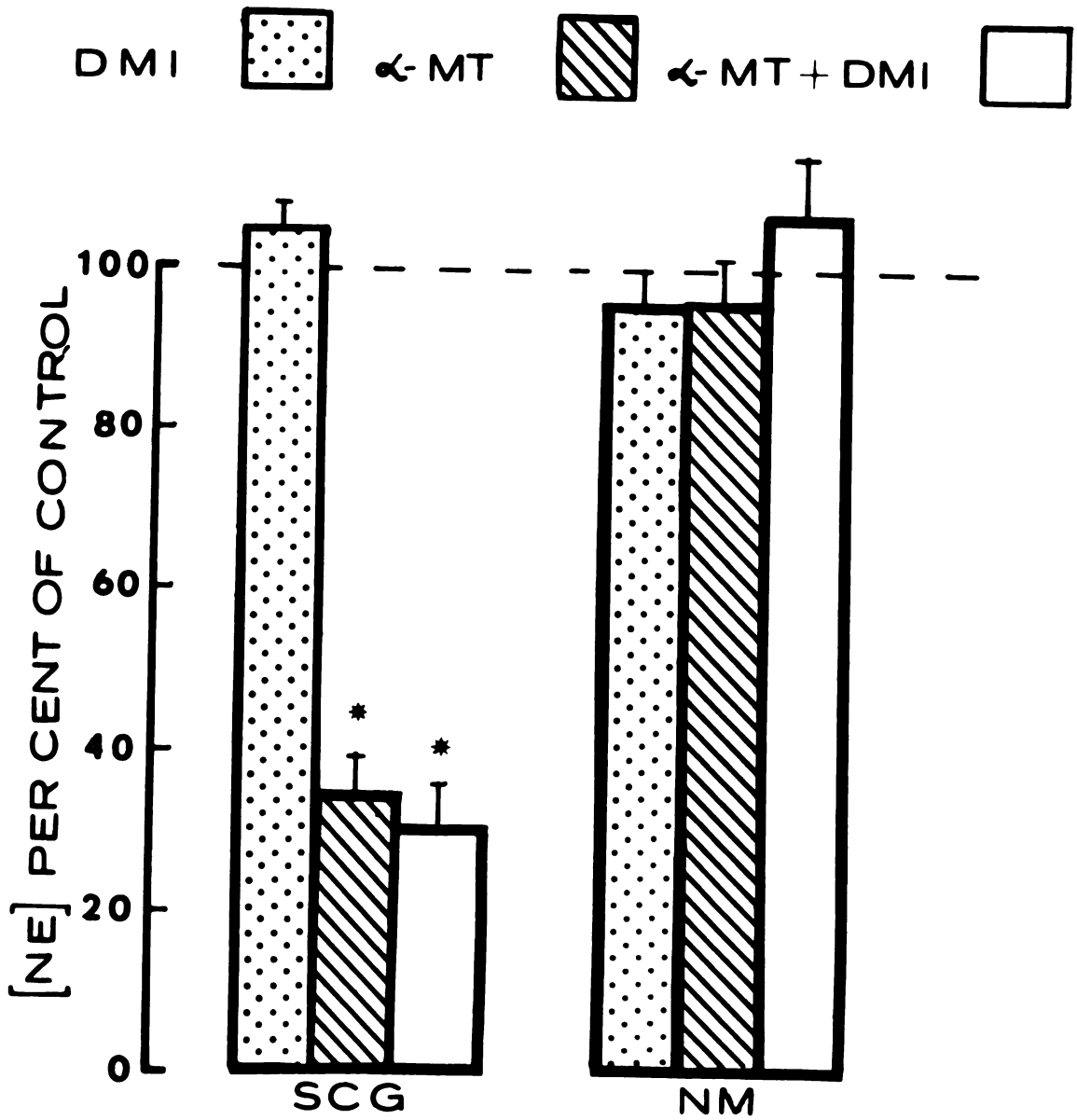

Figure 6. Tissue contents of norepinephrine (NE) following administration of desmethyl-imipramine (DMI) and/or α-methyltyrosine (αMT) in nonstimulated preparations.

Table 3. Tissue norepinephrine (NE) content after three hours of α-methyltyrosine (αMT) infusion in presence of desmethylimipramine (DMI).

Treatment	Ganglia			Salivary glands			Nictitating membranes		
	Control (μg/g)	αMT	% of Control	Control (μg/g)	αMT	% of Control	Control (μg/membrane)	αMT	% of Control
DMI									
2 mg/kg[a]	8.97± 1.29	2.71± .41	31±[c] 5	1.73± .13	1.68± .04	97± 5	1.04± .12	1.12± .14	107± 7
10 mg/kg[b]	6.78± .26	1.74± .54	25±[c] 7	1.77± .19	1.78± .22	101± 8	0.71± .04	0.85± .06	121± 2

Control values represent the NE content of tissues that were removed before the start of the αMT and DMI administration. Values represent the mean ± 1 S.E. as determined from 3 separate experiments.

[a]DMI was injected i.v. 30 min before the start of αMT infusion.

[b]DMI was infused i.v. for 3 hours at the rate of 0.055 mg/min concomitant with the start of αMT infusion.

[c]Those percentages that are significantly different from 100 ($P<.01$).

B. The Dynamics of Norepinephrine in Neuronal Cell Bodies and Terminals During Electrical Stimulation.

1. Effects of stimulation, α-methyltyrosine and desmethylimipramine on tissue contents of norepinephrine

In this series of experiments the tissues of both sides received 0.9% sodium chloride, α-methyltyrosine, and/or desmethylimipramine but the sympathetic trunk of only one side was stimulated preganglionically. The nonstimulated tissues served as the appropriate controls.

a. Effect of stimulation

In the absence of drugs the norepinephrine contents in cell bodies were not altered by continuous (60 sec/min) or intermittent (30 sec/min) preganglionic nerve stimulation for 1-3 hours at low (2 hz) or at high (10 hz) frequencies (Tables 4-6; Figures 7 and 9).

In the terminals, the norepinephrine contents were not significantly altered by continuous (Table 4) or intermittent (Table 5) stimulation at low frequencies, but at high frequencies of intermittent stimulation for 1 hour the norepinephrine contents in the salivary glands and nictitating membranes were reduced to 36% and 78% of control respectively (Table 6). Similar reductions of norepinephrine content were obtained when continuous stimulation was utilized (Table 6). The reduced concentrations of norepinephrine were maintained for 3 hours of stimulation (Table 7; Figure 9).

b. Effects of stimulation and α-methyltyrosine

In the cell bodies, the norepinephrine after infusions of two concentrations (0.05 mg/ml and 0.1 mg/ml) of α-methyltyrosine was not altered by continuous pre- or postganglionic stimulation at low frequencies (Table 4). Similar results were obtained with high frequencies of intermittent stimulation for 1 or 3 hours (Figure 9) or with low frequencies for 3 hours (Table 5). That is, α-methyltyrosine alone reduced the norepinephrine concentration of cell bodies; the magnitude of this reduction was the same in the presence or absence of stimulation.

In salivary glands, α-methyltyrosine enhanced the reduction in norepinephrine contents following 3 hours of intermittent stimulation at 2 and 10 hz (57% and 12% of control respectively; Table 5 and Figure 7). The reduction in the concentrations of norepinephrine was obtained after 1 hour of stimulation at 10 hz and was maintained for 3 hours of stimulation (Figure 9; Table 7).

c. Effects of stimulation and desmethylimipramine

In the cell bodies, the norepinephrine contents were not altered by desmethylimipramine in the presence or absence of preganglionic stimulation (Tables 5, 7 and 8).

In salivary glands, desmethylimipramine enhanced the stimulus-induced depletion of norepinephrine

Table 4. Effects of stimulation and 5 1/4 hours of α-methyltyrosine (αMT) infusion on tissue contents of norepinephrine (NE).

Treatment	n	Ganglia (μg/g)			Nictitating membranes (μg/membrane)		
		NS	S	$\frac{S}{NS}$ x 100	NS	S	$\frac{S}{NS}$ x 100
Preganglionic stimulation							
Saline	5	8.87± .78	9.82± .85	111	1.13± .14	1.02± .13	90
αMT							
0.05 mg/min	3	3.58± .85	4.37± 1.54	122	1.18± .41	0.96± .39	81
0.10 mg/min	5	2.09± 1.15	1.90± 1.63	91	1.28± .23	0.98± .13	77
Postganglionic stimulation							
αMT							
0.10 mg/min	4	3.71± .71	3.78± .46	102	1.03± .07	0.88± .07	86

Table 4 (Cont'd)

The experiments were carried out in cats in which the preganglionic fibers were sectioned. Postganglionic fibers or the distal end of preganglionic fibers on one side were stimulated (S) continuously at 2 hz. The contralateral side served as nonstimulated (NS) control. Stimulation was interrupted 45 min later for a period of 15 min. After the nictitating membranes had returned to basal levels of tension (7.5 g) both control and experimental membranes were stimulated for 1 min to obtain a test contractile response. Unilateral stimulation of experimental side was resumed and continued for another 45 min period. The stimulation was interrupted 3 more times so that the total stimulation period was 3 hours. Tissues were removed for NE assay 4 1/4 hours after the start of stimulation. Infusion of αMT at the indicated rates was started 1 hour before the start of stimulation. All values represent mean ± 1 S.E.

Figure 7. Tissue contents of norepinephrine (NE) following the administration of desmethylimipramine (DMI) and/or α-methyltyrosine (αMT) in stimulated preparations.

The experiments were carried out in cats in which the preganglionic fibers were sectioned and the distal end stimulated at 10 hz for 3 hours. αMT and DMI were administered according to the schedule described in the legend to Figure 3 beginning at the start of the electrical stimulation. The height of each bar represents the mean content of NE expressed as a percentage of the NE content in the corresponding tissue on the unstimulated side. Asterisks indicate the values that are significantly different from those obtained from animals receiving only saline ($P<.01$). Vertical lines projected on each bar represent 1 S.E. as determined from 4 to 6 experiments. SCG--superior cervical ganglia; SG--salivary glands; NM--nictitating membranes.

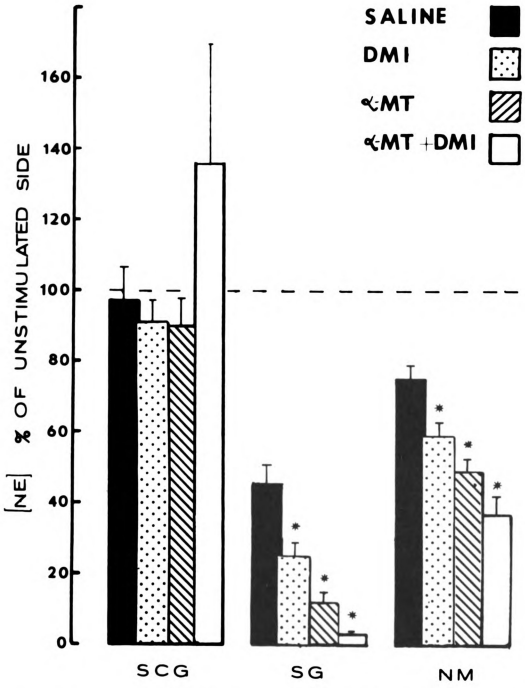

Figure 7. Tissue contents of norepinephrine (NE) following the administration of desmethylimipramine (DMI) and/or α-methyltyrosine (αMT) in stimulated preparations.

Table 5. Effects of α-methyltyrosine (αMT), desmethylimipramine (DMI) and electrical stimulation at 2 and 10 hz on tissue contents of norepinephrine (NE).

Treatment	Frequency of Stimulation	Ganglia (μg/g)		
		NS	S	$\frac{S}{N}$ x 100
Saline	2 hz	5.96± .97	6.92± .93	116
	10 hz	8.25± .81	7.50± .34	91
αMT	2 hz	3.32± .73	3.50± .34	105
	10 hz	3.75± .39	3.37± .52	90
DMI	2 hz	6.59± 1.14	6.40± 1.35	97
	10 hz	9.26± 1.08	8.38± 1.00	90
αMT and DMI	2 hz	2.57± .54	2.60± .77	101
	10 hz	2.06± .12	2.82± .66	137

The experiments were carried out in cats in which the preganglionic fibers were sectioned and the distal end stimulated intermittently (30 sec/min) for 3 hours. αMT was infused through both common carotid arteries at a rate of 0.1 mg/min for 3 hours. DMI (2 mg/kg) was injected i.v. over a period of 5 min concomitant with the start of αMT infusion; another injection of DMI (1 mg/kg) was made two hours later. Values represent the mean content of NE ± 1 S.E. determined in 3 to 7 separate experiments. NS = nonstimulated; S = stimulated.

[a]Significantly different from corresponding saline controls ($P<.05$).

[b]Significantly different from 100% ($P<.05$).

Table 5 (Cont'd)

Salivary Glands (µg/g)			Nictitating Membranes (µg/membrane)		
NS	S	$\frac{S}{N}$ x 100	NS	S	$\frac{S}{N}$ x 100
1.27± .16	1.02± .09	81	1.37± .22	1.28± .25	93
1.70± .30	0.72± .03	42[b]	1.22± .08	0.90± .09	74[b]
1.89± .23	1.07± .17	57[a,b]	1.01± .17	0.76± .17	75
1.29± .10	0.16± .05	12[a,b]	1.08± .13	0.52± .03	48[a,b]
1.47± .31	1.04± .22	71[b]	1.24± .12	1.04± .18	84
1.63± .16	0.39± .05	24[a,b]	1.63± .27	0.98± .19	60[a,b]
1.30± .17	0.58± .08	45[a,b]	0.92± .12	0.80± .18	87
1.70± .18	0.04± .00	2[a,b]	1.26± .11	0.47± .10	37[a,b]

Table 6. Effects of continuous and intermittent stimulation on tissue contents of norepinephrine (NE).

Stimulation	Ganglia (μg/g)		
	NS	S	S/NS x 100
Continuous			
1 hour	6.89± .25	9.17± 1.28	132± 14
3 hours	9.58± .90	9.36± .82	98± 5
Intermittent			
1 hour	10.35± .95	11.56± .84	113± 7
3 hours	8.25± .81	7.50± .34	94± 8

The experiments were carried out in cats in which the preganglionic fibers were sectioned and the distal end stimulated at 10 hz either continuously (60 sec/min) or intermittently (30 sec/min). All values represent the mean content of NE ± 1 S.E. determined in 3 to 5 separate experiments. NS = nonstimulated; S = stimulated.

[a]Significantly different from 100% ($P<.05$).

Table 6 (Cont'd)

Salivary Glands (µg/g)			Nictitating Membranes (µg/membrane)		
NS	S	S/NS x 100	NS	S	S/NS x 100
0.92± .22	0.32± .07	36± 3 [a]	1.45± .25	1.15± .24	78± 4 [a]
1.81± .19	0.63± .08	46± 4 [a]	1.15± .08	0.82± .08	71± 3 [a]
1.55± .27	0.65± .15	42± 4 [a]	1.74± .22	1.44± .15	84± 5
1.70± .30	0.72± .03	45± 6 [a]	1.22± .08	0.72± .09	74± 4 [a]

Table 7. Effects of α-methyltyrosine (αMT), desmethylimipramine (DMI) and stimulation for one and three hours on tissue contents of norepinephrine (NE).

	Saline		αMT	
	1 hr (4)	3 hr (5)	1 hr (4)	3 hr (4)
Ganglia (μg/g)				
NS	10.35± .95	8.25± .81	4.01± .93	3.75± .39
S	11.56± .84	7.50± .34	5.03± 1.34	3.37± .52
S/NS x 100	113± 7	94± 8	127± 19	88± 8
Salivary glands (μg/g)				
NS	1.55± .27	1.70± .30	1.49± .18	1.29± .10
S	0.65± .15	0.72± .03	0.35± .10	0.16± .05
S/NS x 100	42±[b] 4	45±[b] 6	22±[a,b] 5	12±[a,b] 3
Nictitating membranes (μg/memb)				
NS	1.74± .22	1.22± .08	1.57± .12	1.08± .13
S	1.44± .15	0.72± .09	1.18± .10	0.52± .03
S/NS x 100	84± 5	74±[b] 4	75±[b] 3	49±[a,b] 4

The experiments were carried out in cats in which the preganglionic fibers were sectioned and the distal end stimulated intermittently (30 sec/min) at 10 hz. NS = nonstimulated; S = stimulated. αMT was infused through both common carotid arteries at a rate of 0.1 mg/min for 1 or 3 hours. DMI

Table 7 (Cont'd)

DMI		αMT and DMI	
1 hr (3)	3 hr (5)	1 hr (5)	3 hr (6)
9.07± .63	9.26± 1.08	6.16± 1.12	2.06± .12
10.30± .49	8.38± 1.00	6.64± 1.38	2.82± .66
114± 4	92± 6	106± 5	138± 32
1.50± .14	1.63± .16	1.62± .18	1.70± .18
0.27± .07	0.39± .05	0.10± .02	0.04± 0
18±[a,b] 4	25±[a,b] 4	6±[a,b] 1	3±[a,b] 0
1.04± .05	1.63± .27	1.21± .13	1.26± .11
0.70± .08	0.98± .19	0.88± .10	0.47± .10
69±[b] 11	58±[a,b] 4	72±[a] 1	37±[a,b] 6

(2 mg/kg) was injected i.v. over a 5 min period concomitant with the start of the αMT infusion and stimulation; another injection of DMI (1 mg/kg) was made 2 hours later. Values represent the mean ± 1 S.E., figures in parentheses = N.

[a]Values that are significantly different from saline controls and 100% (P<.05).

[b]Values that are significantly different from 100% (P<.05).

Figure 8. Effect of desmethylimipramine (DMI) on the contractile response of nictitating membranes.

Top panel represents the response of the left nictitating membrane to preganglionic stimulation at 10 hz every 30 seconds. DMI (2 mg/kg, i.v.) was injected at the time marked by arrow. The middle panel represents the baseline tension of right nictitating membrane. The bottom panel represents the blood pressure.

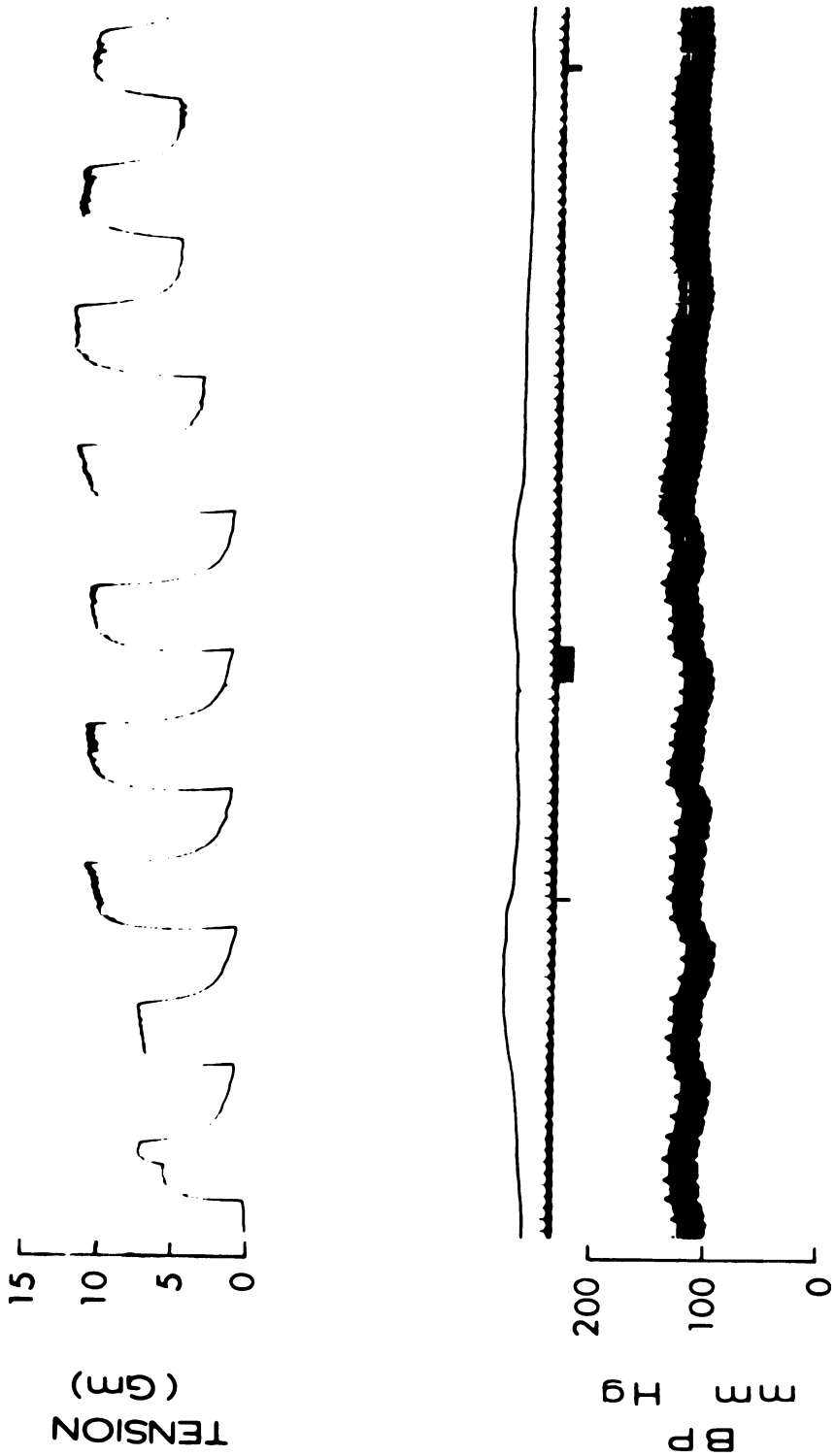

Figure 8. Effect of desmethylimipramine (DMI) on the contractile response of nictitating membranes.

Table 8. Effects of stimulation and desmethylimipramine (DMI) on tissue contents of norepinephrine (NE).

Treatment	Ganglia	Salivary glands	Nictitating membranes
Saline (5)	94±8	41±4	74±4
DMI			
3 mg/kg (5)	91±6	25±4[a]	59±4[a]
6 mg/kg (4)	110±4	20±5[a]	67±4
12 mg/kg (3)	94±8	24±8	64±10

Two-thirds of the indicated dose of DMI was injected i.v. concomitant with the start of stimulation and the remaining one-third of the dose was administered two hours later. The values are the mean ± S.E. expressed as the percent of the unstimulated control content of NE. NE values were determined 3 hours after the start of intermittent preganglionic stimulation at 10 hz. Figures in parentheses represent the number of experiments.

[a]Values are significantly lower than in saline-treated preparations ($P<.05$).

Table 9. Effect of stimulation and desmethylimipramine (DMI) on uptake of norepinephrine-^{3}H (NE-^{3}H).

Tissue	Saline			DMI		
	NS	S	$\frac{S}{NS}$ x 100	NS	S	$\frac{S}{NS}$ x 100
Ganglia (dpm/10 mg)	8,999± 5,683	9,250± 4,348	103± 112	1,365± 1,062	2,162± 650	158± 234
Salivary glands (dpm/g)	198,596± 100,210	601,684± 359,010	303± 49	251,069± 122,070	146,283± 138,200	58±[a] 33
Nictitating membrane (dpm/membrane)	49,214± 23,490	33,696± 21,190	68± 12	25,380± 8,340	3,406± 1,376	13± 14

The experiments were carried out in cats in which the preganglionic fibers were sectioned and the distal end stimulated intermittently (30 sec/min) at 10 hz. NS=nonstimulated, S=stimulated. 10 μc of NE-^{3}H was infused through each common carotid artery during the last one-half hour of a 3-hour stimulation period. DMI (2 mg/kg) was injected i.v. concomitant with the start of stimulation and another 1 mg/kg was administered 1 hour later. Values represent the mean ± 1 S.E. as determined from 3 separate experiments.

[a]This value is significantly different from that obtained after saline treatment ($P<.05$).

only at the high frequency (24% of control; Table 5). The responses obtained with 3, 6 and 12 mg/kg of desmethylimipramine were the same (Table 8). That is, a maximum response was obtained at the lowest dose used (3 mg/kg). As was the case with α-methyltyrosine, the stimulus-induced reduction in norepinephrine content was obtained as early as 1 hour after the start of stimulation and was maintained for 3 hours of stimulation (Figure 9). Both α-methyltyrosine and desmethylimipramine enhanced the stimulus-induced reduction to approximately the same extent (20% of control; Figure 9).

In these experiments desmethylimipramine was utilized to block the retrieval of norepinephrine. It is possible that the observed effects may have resulted as a consequence of some other actions of the drug (Brodie *et al.*, 1968). Nevertheless, as illustrated in Figure 8 and Table 9, desmethylimipramine blocked the uptake of stimulus-induced release of norepinephrine into the noradrenergic terminals.

Norepinephrine-^{3}H accumulated in both neuronal cell bodies and terminals (Table 9). In saline-treated cats, stimulation alone did not increase the uptake of norepinephrine-^{3}H in the ganglia, but caused a 3-fold increase in salivary glands. There was a marked decrease in uptake of norepinephrine-^{3}H in the nictitating membranes; this was probably due to the reduced blood

Figure 9. Effects of α-methyltyrosine (αMT), desmethylimipramine (DMI) and stimulation on the norepinephrine (NE) contents of ganglia, salivary glands and nictitating membranes.

Decentralized preganglionic fibers were stimulated unilaterally for 1 or 3 hours. Contralateral nonstimulated tissues served as controls. 0.9% sodium chloride solution (●) was infused i.a. at a rate of 0.1 ml/min; DMI (■) 2 mg/kg was injected i.v. concomitant with the start of stimulation. When stimulation was continued for 3 hours, an additional dose of DMI (1 mg/kg) was injected 2 hours after the initial dose. αMT (▲) alone or in combination with DMI (0) was infused through both common carotid arteries for 1 or 3 hours at a rate of 0.1 mg/min. Each point represents the mean of 3 to 6 experiments. The vertical bars represent ± 1 S.E. of the mean. The collated NE contents of nonstimulated ganglia, salivary glands and nictitating membranes were 8.98 ± .63 μg/g, 1.57 ± .06 μg/g and 1.35 ± .06 μg/ membrane respectively. Single asterisks indicate values that are significantly different from appropriate saline controls and 100% ($P<.05$). Double asterisks indicate values that are significantly less than 100 ($P<.05$).

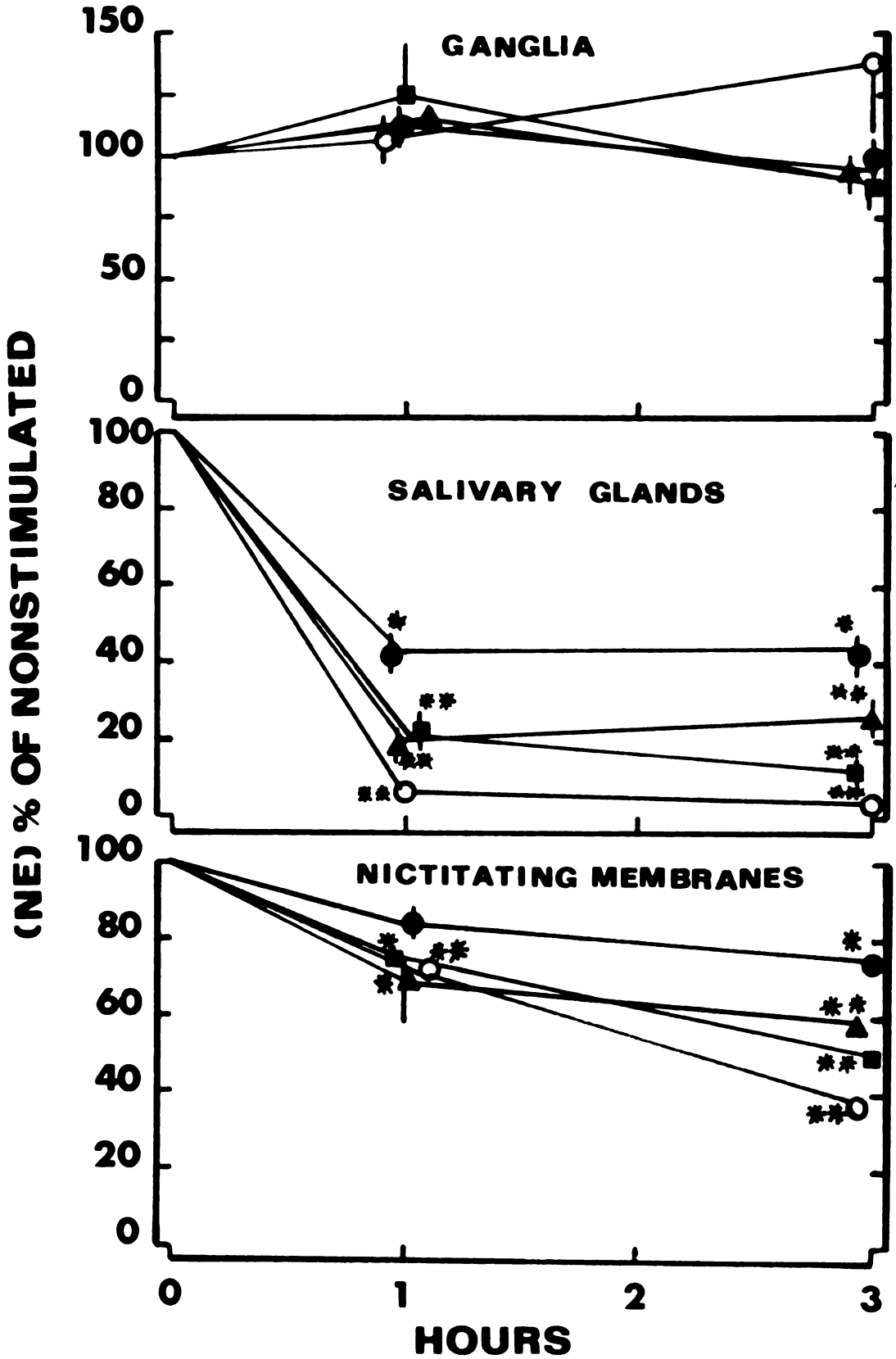

Figure 9. Effects of α-methyltyrosine (αMT), desmethylimipramine (DMI) and stimulation on the norepinephrine (NE) contents of ganglia, salivary glands and nictitating membranes.

flow resulting from contraction of vascular smooth muscles during stimulation periods. Desmethylimipramine did not alter the uptake of norepinephrine-^{3}H in the stimulated ganglia, but caused a 5-fold reduction in the incorporation of norepinephrine in stimulated salivary glands and nictitating membranes.

The effect of desmethylimipramine on the stimulus-induced contractile response of the nictitating membranes is illustrated in Figure 8. Immediately following the administration of desmethylimipramine there was a slight increase in the contractile response of the nictitating membranes and a marked slowing in the rate of relaxation. From Figure 8, it appears that the initial rate of relaxation is not altered by desmethylimipramine and that the resting tension is increased. Nevertheless, if the membranes were allowed to relax after a single stimulus the tension returned to pre-stimulus levels within 3 min in saline-treated cats, but it took approximately 9 min in desmethylimipramine-treated cats. These results suggest a more prolonged contact of released norepinephrine with the receptor as a result of reduced uptake of this amine. Similar results have been reported by Fischer and Snyder (1965) using cocaine, and by Sigg *et al.* (1963) using desmethylimipramine to block norepinephrine uptake.

d. Effects of stimulation, α-methyltyrosine and desmethylimipramine

In the presence of a combination of α-methyltyrosine and desmethylimipramine the effects of various schedules of stimulation on the norepinephrine content of cell bodies were the same as those obtained in the presence of α-methyltyrosine alone (Table 5). That is, the α-methyltyrosine-induced reduction in the ganglionic contents of norepinephrine was not altered by desmethylimipramine in the presence or absence of stimulation.

In the salivary glands, the combination of desmethylimipramine and α-methyltyrosine enhanced the norepinephrine depletion resulting from 3 hours of preganglionic stimulation at 2 hz to a greater extent than that produced by either one of these drugs alone (Table 5). Stimulation at 10 hz in the presence of α-methyltyrosine and desmethylimipramine, almost totally depleted norepinephrine within 1 hour (Tables 5 and 7; Figure 7).

The effects of stimulation, α-methyltyrosine and desmethylimipramine in the nictitating membranes were qualitatively similar to those seen in the salivary glands, with the changes in norepinephrine concentrations being less pronounced; the effects were significant only at higher frequency of stimulation.

For example, stimulation for 1 hour alone, or in the presence of α-methyltyrosine and/or desmethylimipramine, did not cause a marked reduction in the norepinephrine content of nictitating membranes (Figure 9; Table 7); the effects of stimulation, α-methyltyrosine and desmethylimipramine were not evident until after 3 hours of stimulation.

These results suggest that in cell bodies norepinephrine concentrations are maintained by synthesis independent of the frequency of neuronal activity. In nerve terminals, stimulation reduces the norepinephrine concentrations to new steady state levels; synthesis partially maintains the norepinephrine concentrations during high and low frequencies of stimulation. Reuptake, on the other hand, appears to play a significant role only at higher frequencies of stimulation.

2. Effects of preganglionic stimulation and α-methyltyrosine on the conversion of tyrosine-^{14}C to norepinephrine-^{14}C

The formation of norepinephrine-^{14}C in cell bodies and terminals of stimulated and nonstimulated neurons was determined following i.a. administration of tyrosine-^{14}C (Table 10).

Following i.a. infusions of tyrosine-^{14}C relatively more of this amino acid was converted to norepinephrine in resting or nonstimulated cell bodies than in the corresponding terminals. Thus, in ganglia

Table 10. Effect of stimulation and α-methyltyrosine (αMT) on the conversion of i.a. administered tyrosine-^{14}C to norepinephrine-^{14}C (NE-^{14}C).

	Total radioactivity			NE-^{14}C			$\frac{\text{NE-}^{14}\text{C}}{\text{Total radioactivity}}$ x 100		
	NS	S	$\frac{S}{NS}$	NS	S	$\frac{S}{NS}$	NS	S	$\frac{S}{NS}$
Ganglia (dpm/10 mg)									
Saline	1,351± 805	1,748± 295	1.29	270± 191	269± 102	1.00	16.80± 2.70	14.30± 3.20	0.85
αMT	1,014± 102	1,078± 133	1.06	23± 6	52± 13	2.26[a]	2.20± .60	4.90± 1.50	2.22
Salivary glands (dpm/g)									
Saline	36,789± 8,663	74,215± 16,014	2.02[a]	55± 15	1,225± 153	22.27[a]	0.15± .02	1.73± .18	11.53[a]
αMT	47,159± 12,696	53,047± 12,401	1.12	16± 6	29± 4	1.81	0.04± .02	0.06± .01	1.50
Nictitating membrane (dpm/memb)									
Saline	64,760± 11,772	39,080± 5,904	0.60[a]	61± 13	196± 14	3.21	0.09± 0	0.50± .07	5.56[a]
αMT	53,400± 9,856	66,080± 6,723	1.24	16± 4	44± 4	2.75[a]	0.03± 0	0.07± 0	2.33[a]

Table 10 (Cont'd)

25 μc of tyrosine-^{14}C was infused through each common carotid artery during the last one-half hour of a 3-hour stimulation period. αMT was infused concomitant with the start of stimulation at the rate of 0.1 mg/min for 3 hours. Decentralized preganglionic fibers were stimulated intermittently at 10 hz. NS = nonstimuled, S = stimulated. Values represent the mean ± 1 S.E. as determined from 3 separate experiments.

[a]These values are significantly different from 1 ($P<.05$).

approximately 17% of the total radioactivity was represented by norepinephrine whereas in salivary glands and nictitating membranes norepinephrine accounted for only 0.1% of total radioactivity. These results would be expected if the turnover of norepinephrine is faster in the cell bodies. Preganglionic stimulation markedly increased the formation of norepinephrine-^{14}C in tissues containing nerve terminals; the norepinephrine-^{14}C content in stimulated salivary glands was 22 times greater, and in nictitating membranes was 3 times greater than in the respective nonstimulated tissues. This increase is also apparent when the norepinephrine-^{14}C is expressed as a percent of total radioactivity. Stimulation caused an approximate 12 fold increase in the percent norepinephrine-^{14}C in salivary glands and a 5 fold increase in nictitating membranes. On the other hand, there was no difference between the amount of norepinephrine-^{14}C in terms of absolute amount or percent of total radioactivity, in stimulated and nonstimulated ganglia, when tyrosine-^{14}C was infused i.a. (Table 10).

Treatment with α-methyltyrosine did not alter total radioactivity but reduced the conversion of tyrosine-^{14}C to norepinephrine-^{14}C in all tissues (Table 10). In nonstimulated preparations the effect of α-methyltyrosine was more pronounced in cell bodies (a 10 fold reduction)

than in terminals (a 3 fold reduction in both salivary glands and nictitating membranes). In salivary glands and nictitating membranes α-methyltyrosine caused a relatively greater inhibition of norepinephrine-^{14}C formation in stimulated than in nonstimulated tissues; this was not true in ganglia. Interpretation of these latter results, however, should be tempered by the fact that after α-methyltyrosine the radioactivity of norepinephrine in all tissues was very low, less than twice the background.

To avoid possible errors that might result from the i.a. administration of tyrosine-^{14}C (e.g., unequal distribution resulting from changes in blood flow due to surgical manipulations in the neck region) experiments were performed using i.v. administration of this amino acid. The results obtained with i.v. and i.a. infusion of tyrosine-^{14}C were similar (compare Tables 10 and 11). Following i.v. tyrosine-^{14}C more norepinephrine-^{14}C accumulated in the nonstimulated ganglia (9.8% of total radioactivity) than in nonstimulated salivary glands and nictitating membranes (approximately 0.1% of total radioactivity). Stimulation markedly increased the formation of norepinephrine-^{14}C in the latter two tissues but did not significantly influence the amount of this radioactive amine in the ganglia.

Table 11. Effect of stimulation on the conversion of i.v. administered tyrosine-^{14}C to norepinephrine-^{14}C (NE-^{14}C).

	Total radioactivity			NE-^{14}C			$\frac{\text{NE-}^{14}\text{C}}{\text{Total radioactivity}} \times 100$		
	NS	S	$\frac{S}{NS}$	NS	S	$\frac{S}{NS}$	NS	S	$\frac{S}{NS}$
Ganglia (dpm/10 mg)	1,357± 201	1,604± 197	1.18	124± 17	213± 30	1.72[a]	9.80± 1.9	13.30± 1.5	1.36
Salivary glands (dpm/g)	38,271± 6,889	60,710± 7,018	1.59[a]	43± 10	839± 242	19.51[a]	0.11± 0	1.33± .28	12.09[a]
Nictitating membranes (dpm/memb)	55,710± 8,414	52,620± 9,000	0.94	31± 3	259± 69	8.35[a]	0.06± .01	0.54± .16	9.00[a]

100 µc of tyrosine-^{14}C was infused through a femoral vein during the last one-half hour of a 3-hour stimulation period. Decentralized preganglionic fibers were stimulated intermittently at 10 hz (NS = nonstimulated; S = stimulated). Values represent the mean ± 1 S.E. as determined from 4 separate experiments.

[a]These values are significantly different from 1 ($P<.05$).

3. Effects of stimulation and cycloheximide on the tissue contents of norepinephrine and on the conversion of tyrosine-^{14}C to norepinephrine-^{14}C

Previous results indicated that stimulation does not modify the norepinephrine contents or the synthesis of norepinephrine in cell bodies but increases the synthesis of this amine in nerve terminals. In cell bodies, synthesis of norepinephrine may proceed at a maximal rate which cannot be modified by acute stimulation. In the terminals, part of the stimulus-induced augmentation in norepinephrine synthesis could be due to an increase in catecholamine synthesizing enzymes. These possibilities were tested with the administration of cycloheximide, a drug which blocks protein synthesis. The results are summarized in Tables 12-14.

Cycloheximide increased the contents of norepinephrine in nonstimulated ganglia and nictitating membranes (Table 12). This compound did not modify the stimulus-induced decline of norepinephrine in salivary glands and nictitating membranes (Table 13). In salivary glands the norepinephrine contents were 45 and 41% of control and in the nictitating membranes 74 and 88% of control in the absence and presence of cycloheximide respectively. When tyrosine-^{14}C was infused the total radioactivity in all tissues was doubled in the presence of cycloheximide (Table 14). Because of the variability,

Table 12. Effect of cycloheximide on tissue contents of norepinephrine (NE).

Ganglia (µg/g)			Salivary Glands (µg/g)			Nictitating Membranes (µg/membrane)		
Control	Cyclo-heximide	% of Control	Control	Cyclo-heximide	% of Control	Control	Cyclo-heximide	% of Control
7.53± .71	10.58± .80	141±[a] 4	1.76± .20	1.96± .19	113± 12	1.26± .17	1.48± .12	144±[a] 16

Control values represent the NE content of tissues that were removed before the administration of cycloheximide (10 mg/kg, i.p.); treated tissues were removed 4 hours later. Values represent the mean NE content ± 1 S.E. as determined from 3 separate experiments.

[a]Values are significantly different from 100% ($P<.05$).

Table 13. Effects of stimulation and cycloheximide on tissue contents of endogenous norepinephrine.

Treatment	Salivary Glands (µg/g)			Nictitating Membranes (µg/membrane)		
	NS	S	$\frac{S}{NS} \times 100$	NS	S	$\frac{S}{NS} \times 100$
None	1.70± .30	0.72± .03	45± 6 [a]	1.22± .08	0.72± .09	74± 4 [a]
Cycloheximide	1.36± .15	0.58± .19	41± 9 [a]	1.09± .05	0.96± .10	88± 5

The experiments were carried out in cats in which the preganglionic fibers were sectioned and the distal end stimulated at 10 hz for 3 hours (NS = nonstimuled; S = stimulated). Cycloheximide (10 mg/kg) was injected i.p. one hour before the start of stimulation. Values represent the mean ± 1 S.E. as determined from three separate experiments.

[a]Values that are significantly different from 100 ($P<.05$).

Table 14. Effect of stimulation and cycloheximide on the conversion of i.a. administered tyrosine-^{14}C to norepinephrine-^{14}C (NE-^{14}C).

	Total radioactivity			NE-^{14}C			$\frac{\text{NE-}^{14}\text{C}}{\text{Total radioactivity}} \times 100$		
	NS	S	$\frac{S}{NS}$	NS	S	$\frac{S}{NS}$ +	NS	S	$\frac{S}{NS}$
Ganglia (dpm/10 mg)	4,744± 2,790	20,028± 17,705	4.22	379± 165	798± 425	2.11	9.15± 3.51	11.60± 4.44	1.27
Salivary glands (dpm/g)	71,639± 35,868	175,067± 108,021	2.44	101± 40	855± 563	8.46[a]	0.16± .01	0.47± .06	2.94[a]
Nictitating membranes (dpm/membrane)	110,240± 13,927	88,920± 19,827	0.81	117± 25	204± 45	1.74	0.11± .01	0.23± .04	2.01[a]

25 μc of tyrosine-^{14}C was infused through each common carotid artery during the last one-half hour of a 3-hour stimulation period. Decentralized preganglionic fibers were stimulated intermittently at 10 hz. NS = nonstimulated; S = stimulated. Cycloheximide (10 mg/kg) was injected i.p. one hour before the start of stimulation. Values represent the mean ± 1 S.E. as determined from three separate experiments.

[a]These values are significantly different from 1 ($P<.05$).

total radioactivity and the absolute amounts of norepinephrine-^{14}C were not statistically different in the stimulated and nonstimulated sides. Norepinephrine-^{14}C, reported as a percent of total radioactivity, was the same in stimulated and nonstimulated ganglia, but was increased 3 and 2 fold in stimulated salivary glands and nictitating membranes respectively. This increase was much less than that obtained in the absence of cycloheximide (compare Tables 10 and 14). The variability in these experiments prohibited definite conclusion, but it appears that cycloheximide does not affect the formation of norepinephrine-^{14}C in cell bodies but reduces the stimulus-induced increase in the formation of norepinephrine-^{14}C in the nerve terminals.

4. Possible fate of norepinephrine in cell bodies

Norepinephrine in the cell bodies may be released, metabolized by monoamine oxidase, and/or transported down the axon. Pretreatment with a monoamine oxidase inhibitor (pheniprazine) did not alter significantly the α-methyltyrosine-induced depletion of norepinephrine in the ganglia (Table 15). Pheniprazine (5 mg/kg, i.v.) was administered to two cats. One hour later the ganglia on one side were excised and α-methyltyrosine was then infused through the common carotid arteries (0.1 mg/min for 3 hours). The norepinephrine content declined to 41% of control

Table 15. Effects of pheniprazine and three hours of α-methyltyrosine (αMT) infusion on the tissue contents of norepinephrine (NE).

Treatment		Ganglia (μg/g)			Salivary Glands (μg/g)		
		Control	αMT	% of Control	Control	αMT	% of Control
None	(4)	6.74± .41	2.31± .23	35± 4	0.96± .20	0.89± .18	93± 5
Pheniprazine	(2)	7.35± 1.17	3.06± .62	41± 2	1.05± 00	1.15± .02	110± 1

Control values represent the NE content of tissues that were removed before the start of the administration of drugs. Pheniprazine (5 mg/kg) was injected i.v. 1 hour before the start of αMT infusion. Values represent the mean ± 1 S.E. as determined from the number of experiments indicated in the parentheses.

during the α-methyltyrosine infusion. This reduction is similar to that obtained in the absence of a monoamine oxidase inhibitor (see Table 2).

Norepinephrine is transported centrifugally from the cell body. This transport appears to contribute to the rapid decline of norepinephrine in the ganglia following α-methyltyrosine administration. When the postganglionic fibers were tied the depletion of norepinephrine was reduced (Table 16). This experiment was repeated after the administration of pheniprazine; the results were essentially the same as those obtained in the absence of pheniprazine. The interruption of somatofugal transport of axonal constituents for 3 hours did not alter the norepinephrine content of salivary glands (1.06 ± .39 and 1.09 ± .32 μg norepinephrine/g in salivary glands on intact and tied sides respectively).

5. Possible formation of α-methylnorepinephrine from α-methyltyrosine

Some α-methyltyrosine is biotransformed to α-methylnorepinephrine (Udenfriend *et al.*, 1966; Maitre, 1965; Van Orden *et al.*, 1970) which in turn could displace endogenous norepinephrine (Dominic and Moore, 1971). α-Methyltyrosine-induced depletion of norepinephrine in terminals in the presence of α-methyltyrosine, might result from the formation of α-methylnorepinephrine. Therefore, experiments were

Table 16. Effects of α-methyltyrosine (αMT), pheniprazine and tying of postganglionic fibers on the norepinephrine (NE) content of ganglia.

Treatment		Postganglionic Fibers Intact	Postganglionic Fibers Tied	Percent of Intact
αMT, 1 hr	(3)	4.85± 1.03	7.30± 1.93	150± 20
αMT, 3 hrs	(5)	1.87± 0.97	3.77± 1.57	202±[a] 25
αMT, 3 hrs + Pheniprazine	(3)	3.63± 1.20	5.38± 1.20	157±[a] 15

Postganglionic fibers of both sides were exposed and those of one side were tied immediately cranial to the ganglia with a silk thread. αMT was then infused through both common carotid arteries at the rate of 0.1 mg/min. Pheniprazine (5 mg/kg) was injected i.v. one hour before the start of the αMT infusion. Values represent the mean ± 1 S.E. of norepinephrine (μg/g) as determined from the number of experiments indicated in the parentheses.

[a]These values are significantly greater than 100% ($P<.05$).

conducted to determine if the formation of α-methyl-norepinephrine contributed to the actions of α-methyltyrosine in cell bodies and terminals.

Twenty-five μc of α-methyltyrosine-^{3}H was infused through each common carotid artery during the last one-half hour of a three hour stimulation period. The results are summarized in Table 17. In nonstimulated ganglia and salivary glands very little radioactivity was detected in the α-methylnorepinephrine fraction. The counts were too low (less than twice background) to further identify this radioactivity. Furthermore, stimulation did not increase the amount of radioactivity in the α-methylnorepinephrine fraction. This is in contrast to the marked increase in the conversion of tyrosine-^{14}C to norepinephrine-^{14}C in stimulated salivary glands (Table 10 and 11).

6. Norepinephrine contents and the contractile responses of nictitating membranes

   a. Effects of α-methyltyrosine and continuous stimulation at low frequency (2 hz)

α-Methyltyrosine solutions (0.05 mg/ml or 0.1 mg/ml) were infused i.a. for 5 1/4 hours. One hour after the beginning of α-methyltyrosine infusion continuous stimulation of pre- or postganglionic fibers was started. The stimulation was interrupted every 45 min to record a test contractile response of

Table 17. Formation of "apparent α-methylnorepinephrine-^{3}H (αMNE-^{3}H)" after infusion of α-methyltyrosine-^{3}H (αMT-^{3}H).

Tissue	Units	Total radioactivity		"Apparent α-MNE-^{3}H"	
		NS	S	NS	S
Ganglia	dpm/10 mg	712± 57	1,063± 270	43± 15	39± 14
Salivary glands	dpm/g	21,770± 2,399	22,038± 2,808	65± 32	70± 15

25 μc of α-MT-^{3}H was infused through each common carotid artery during the last one-half hour of a three-hour stimulation period. Decentralized preganglionic fibers were stimulated intermittently at 10 hz (NS = nonstimulated; S = stimulated).

All values represent the mean ± 1 S.E. as determined from 3 experiments.

Figure 10. Effects of stimulation and α-methyltyrosine (αMT) on the contractile responses of the nictitating membranes.

The experiments were carried out in cats in which the preganglionic fibers were sectioned. αMT was infused through both common carotid arteries at a rate of 0.05 mg/min or 0.1 mg/min for 5 1/4 hours. One hour after the start of αMT infusion (arrow), postganglionic fibers or the distal end of preganglionic fibers were stimulated continuously at 2 hz. Stimulation was interrupted 45 min later for a period of 15 min. After the nictitating membranes had returned to basal levels of tension (7.5 g) both control and experimental membranes were stimulated for 1 min to obtain a test contractile response. Unilateral stimulation of experimental side was resumed and continued for another 45 min period. The stimulation was interrupted 3 additional times so that the total stimulation period was 3 hours.

Each point represents the mean nictitating membrane contraction expressed as percent of the control contraction that was obtained at appropriate times by 1 min stimulation of the nonstimulated nictitating membranes. Vertical lines projected on each point represent 1 S.E. as determined from 3 to 5 experiments. Preganglionic stimulation (saline 0; αMT 0.05 mg/min ▲; αMT 0.10 mg/min ●). Postganglionic stimulation (αMT 0.10 mg/min ■).

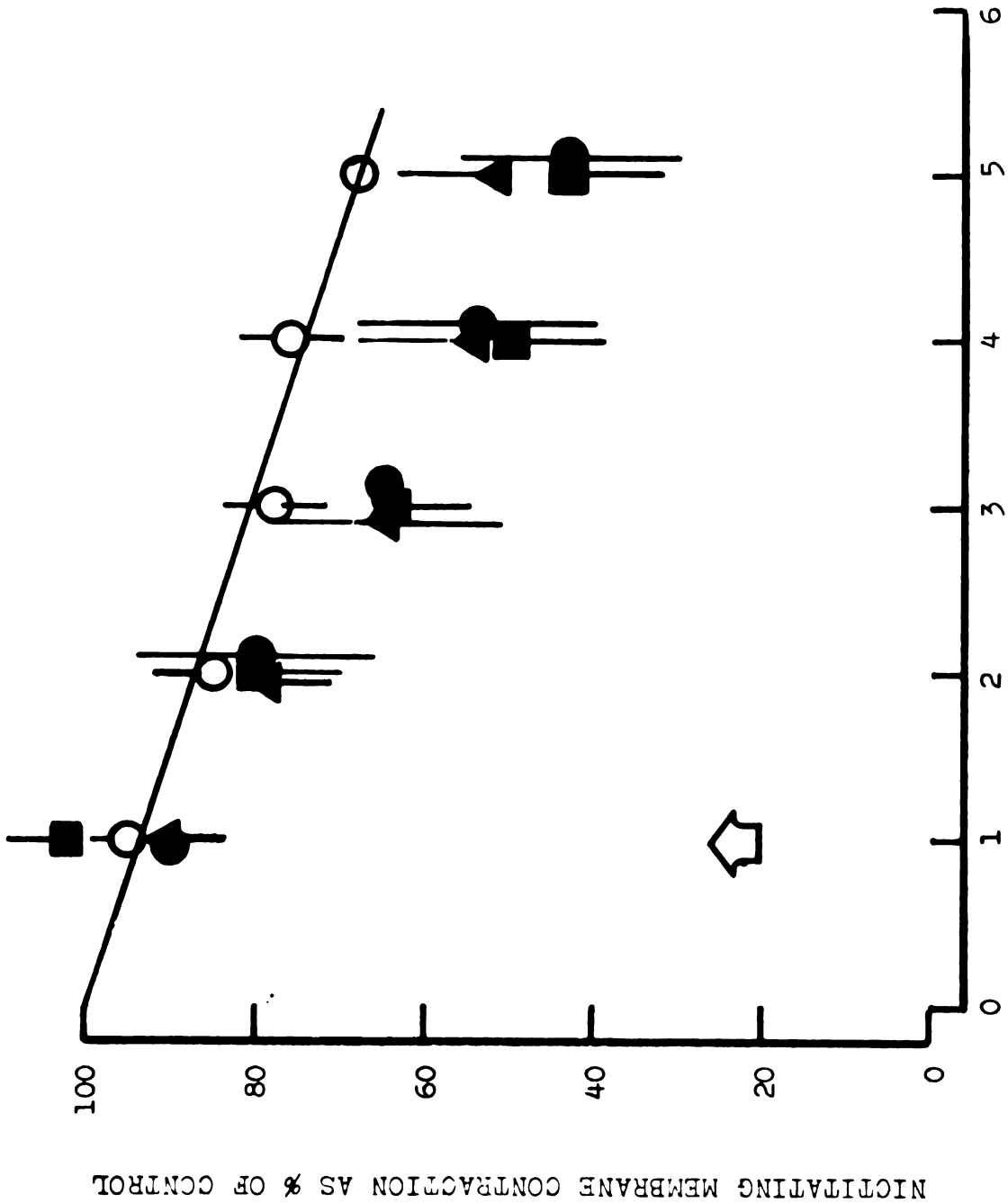

Figure 10. Effects of stimulation and α-methyltyrosine (αMT) on the contractile responses of the nictitating membranes.

nonstimulated and stimulated nictitating membranes. The results are summarized in Figure 10. The contractions of the nictitating membranes progressively decreased in saline-infused (control) cats. Pre- or postganglionic stimulation in the presence of α-methyltyrosine appears to reduce the contractile responses, but the effects were variable and not significantly different from those obtained after stimulation alone. Similarly, there was no significant difference in the norepinephrine contents of nictitating membranes between saline- and α-methyltyrosine-treated cats after pre- or postganglionic stimulation (Table 4).

b. Effects of α-methyltyrosine and continuous stimulation at frequencies of 2 hz, 10 hz, and 16 hz.

Since the contractile responses of nictitating membranes were small and variable at low frequencies, stimulation was carried out at higher frequencies. The results are summarized in Table 18. The contractions of nictitating membranes (expressed as % of initial contractions) in α-methyltyrosine-treated cats at stimulation frequencies of 10 hz and 16 hz were significantly less than those obtained at 2 hz. A significant reduction in norepinephrine contents was obtained only after stimulation at 16 hz. In saline-treated cats, the norepinephrine contents and

Table 18. Effects of stimulation and α-methyltyrosine (αMT) on the norepinephrine (NE) contents and contractile response of nictitating membranes.

Treatment	Frequency of Stimulation	Contractile Response[a] n		NE[b] n	
αMT	2 hz	3	85± 9	3	76± 8
αMT	10 hz	3	56±[c] 3	2	74± 10
αMT	16 hz	3	45±[c] 8	3	69±[c] 6
Saline	16 hz	2	71± 11	3	66± 13

The experiments were carried out in cats in which the preganglionic fibers were sectioned. αMT was infused i.a. at a rate of 0.1 mg/min for 75 min. Fifteen minutes after the start of αMT infusion, decentralized preganglionic fibers on one side were stimulated continuously for 1 hour at the indicated frequencies.

[a]The values are the mean ± 1 S.E. expressed as the percent of the initial contractile response.

[b]The values are the mean ± 1 S.E. expressed as the percent of the nonstimulated control content of NE.

[c]These values are significantly different from 100 ($P<.05$).

contractions of nictitating membranes were not significantly different from controls after 1 hour of stimulation at 16 hz but were reduced in the presence of α-methyltyrosine.

c. Effects of α-methyltyrosine, desmethylimipramine and intermittent stimulation at low (2 hz) and high (10 hz) frequencies.

To further test whether a correlation existed between the norepinephrine contents and contractions of nictitating membranes experiments were conducted after the blockade of both synthesis and uptake of norepinephrine. The results are summarized in Figure 11. After three hours of intermittent stimulation at 2 or 10 hz the contractions of nictitating membranes in saline-treated preparations and in those that were treated with α-methyltyrosine and/or desmethylimipramine were not statistically different. On the other hand, when stimulation was carried out at 10 hz for 1 hour the contractile responses in preparations treated with α-methyltyrosine or α-methyltyrosine and desmethylimipramine were significantly less than those of saline-treated preparations. Desmethylimipramine alone did not modify the contractions of nictitating membranes.

There does not appear to be any relationship between the norepinephrine contents and the magnitude of contraction of the nictitating membranes (compare

Figure 11. Contractile responses of the nictitating membranes following the administration of desmethylimipramine (DMI) and/or α-methyltyrosine (αMT) in stimulated preparations.

The experiments were carried out in cats in which the preganglionic fibers were sectioned and the distal end was stimulated intermittently (30 sec/min) at 2 (Panel 1) or 10 hz (Panel 2) for 3 hours or at 10 hz for 1 hour (Panel 3). αMT was infused through both common carotid arteries at a rate of 0.1 mg/min for 1 or 3 hours. DMI (2 mg/kg) was injected i.v. over a 5 min period concomitant with the start of the αMT infusion. When stimulation was continued for 3 hours another injection of DMI (1 mg/kg) was made 2 hours after the initial injection. The height of each bar represents the mean contraction of nictitating membranes at the end of stimulation period expressed as a percentage of the initial contraction of the nictitating membranes. Vertical lines projected on each bar represent 1 S.E. as determined from 2 to 28 observations. Asterisks mark those values that are significantly different from corresponding saline controls ($P<.05$).

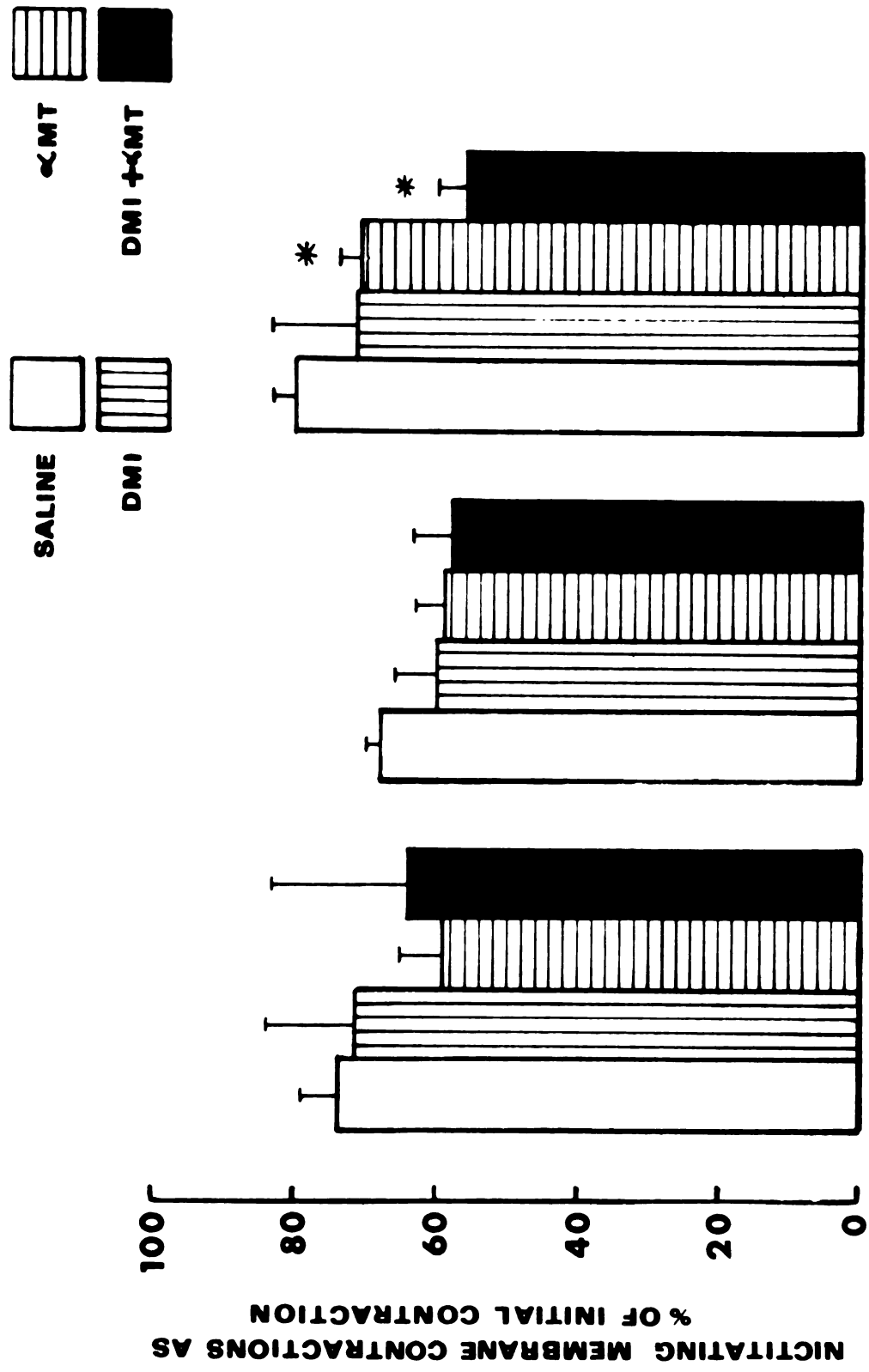

Figure 11. Contractile responses of the nictitating membranes following the administration of desmethylimipramine (DMI) and/or α-methyltyrosine (αMT) in stimulated preparations.

Table 7 and Figure 11). For example, the norepinephrine contents of nictitating membranes following stimulation at 10 hz for 3 hours were reduced to 37% of pre-stimulation values in the presence of α-methyltyrosine + desmethylimipramine whereas in saline-treated preparations the norepinephrine content was only reduced to 74%. However, the contractions of nictitating membranes in saline-treated and α-methyltyrosine + desmethylimipramine-treated preparations were not statistically different. Similar results were obtained by Thoenen *et al.* (1966) who proposed that there was no functional relationship between the content of norepinephrine and the contractions of nictitating membranes.

C. The Dynamics of Norepinephrine in the Neuronal Cell Bodies and Terminals During the Post-Stimulation Period.

1. Restoration of norepinephrine after cessation of stimulation and the effects of desmethylimipramine and α-methyltyrosine

The results of experiments designed to determine the recovery of norepinephrine during the post-stimulation period are summarized in Figure 12 and Table 19. The stimulation-induced depletion of norepinephrine observed in previous experiments was presumably due to the release of norepinephrine from noradrenergic terminals until a new steady state was attained (Figure 9). This

Figure 12. Effects of desmethylimipramine (DMI) and α-methyltyrosine (αMT) on the poststimulation recovery of tissue contents of norepinephrine (NE).

The experiments were carried out in cats in which the preganglionic fibers were sectioned and the distal end stimulated intermittently (30 sec/min) at 10 hz for one hour (Solid bar) or 3 hours (Open bar). Two-thirds of the total dose of DMI (3 mg/kg) was injected i.v. concomitant with the start of stimulation and the remaining one-third 2 hours later. In one set of experiments αMT was infused concomitant with the cessation of stimulation (arrow) through both common carotid arteries at the rate of 0.10 mg/min for 6 hours. Tissues (Ganglia: ▲, saline; Δ, DMI. Salivary glands: ● saline; 0, DMI; □, DMI + αMT) were removed immediately after stimulation and at 2, 4 and 6 hours after the cessation of a 3 hour stimulation period. Each point represents the mean from 3 to 6 experiments and vertical lines projected at each point represent ± 1 S.E.

* Values that are significantly lower than 100% ($P<.05$).

**Values that are significantly different from those obtained immediately after the cessation of a 3 hour stimulation period ($P<.05$) and significantly lower than 100 ($P<.05$).

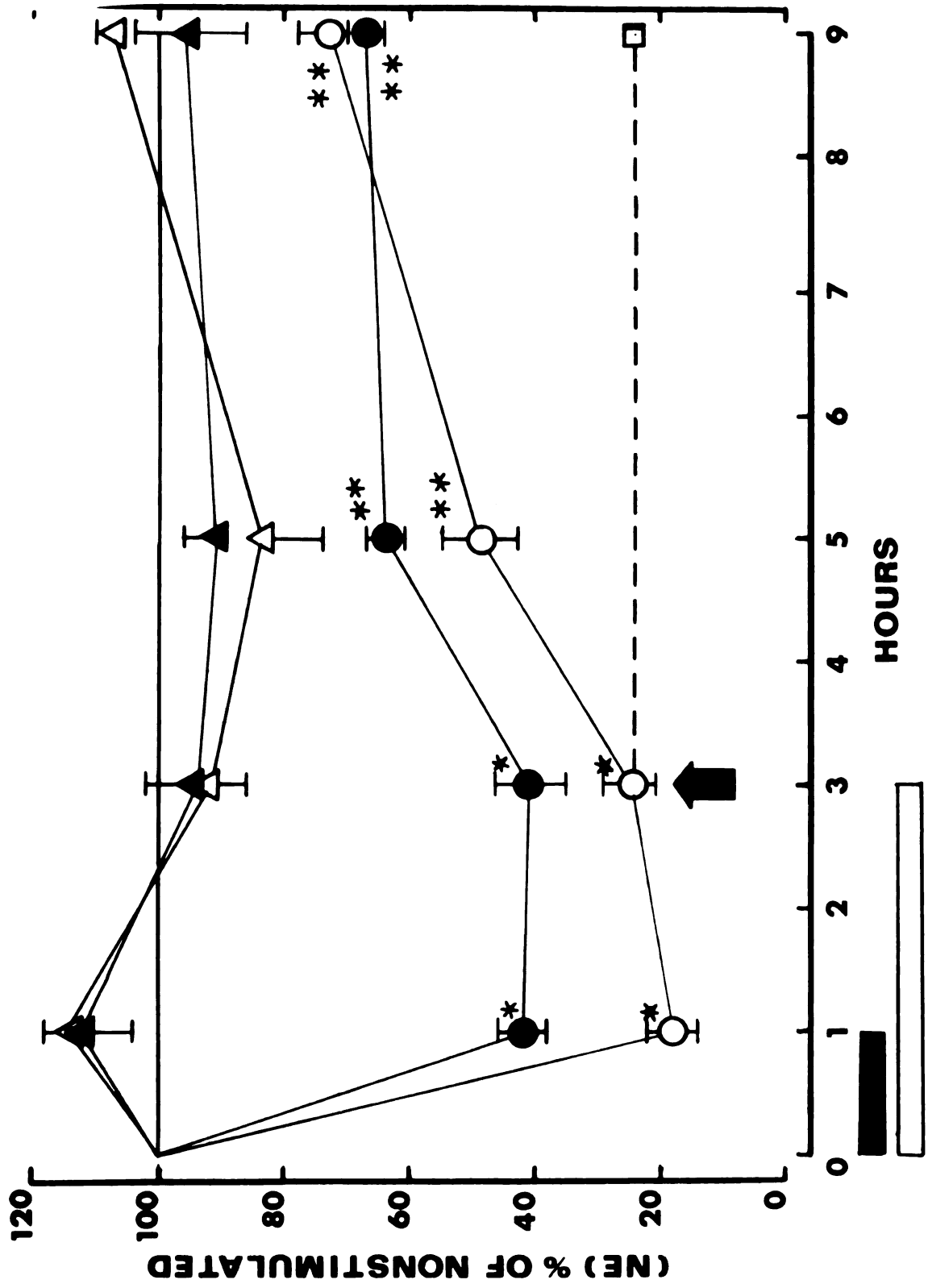

Figure 12. Effects of desmethylimipramine (DMI) and α-methyltyrosine (αMT) on the poststimulation recovery of tissue contents of norepinephrine (NE).

Table 19. Effects of desmethylimipramine (DMI) and α-methyltyrosine (αMT) on the recovery of tissue contents of norepinephrine (NE) following stimulation-induced depletion.

Treatment and time after cessation of stimulation	Ganglia (μg/g)		
	NS	S	S/NS x 100
Saline			
0 hours	8.25± .81	7.50± .34	94
2 hours	11.16± 1.34	10.21± 1.72	91
6 hours	8.61± 1.70	8.06± 1.16	94
DMI			
0 hours	9.26± 1.08	8.38± 1.00	92
2 hours	11.33± 1.24	9.34± 1.06	82
6 hours	6.69± .97	7.20± 1.13	108
DMI and αMT			
6 hours	1.55± .27	1.52± .07	98

The experiments were carried out in cats in which the preganglionic fibers were sectioned and the distal end stimulated intermittently (30 sec/min) at 10 hz for 3 hours. (NS = nonstimulated; S = stimulated). Two-thirds of the total dose of DMI (3 mg/kg) was injected i.v. concomitant with the start of stimulation and the remaining one-third of the dose was injected 2 hours later. αMT was infused through both common carotid arteries at the rate of 0.1 mg/min for 6 hours simultaneously with the cessation of stimulation. Tissue NE contents were determined immediately (0 hour) and 2 and 6 hours after the cessation of stimulation. The tissue contents of NE are reported as the mean ± 1 S.E. as determined from 3 to 5 experiments.

Table 19 (Cont'd)

Salivary Glands (µg/g)			Nictitating membranes (µg/membrane)		
NS	S	S/NS x 100	NS	S	S/NS x 100
1.70± .30	0.72± .03	42[b]	1.22± .08	0.72± .09	74[b]
1.55± .24	0.98± .13	63[b,c]	1.02± .18	0.84± .14	82
1.68± .24	1.10± .13	65[b,c]	1.65± .31	1.47± .42	89
1.63± .16	0.39± .05	25[a,b]	1.63± .27	0.98± .04	58[a]
1.40± .11	0.69± .13	49[b,c]	1.58± .18	1.25± .20	79[b]
1.42± .07	1.04± .04	73[b,c]	1.58± .38	1.12± .10	71
1.35± .33	0.33± .04	24[d]	1.16± .18	0.80± .03	69

[a]These values are significantly different from corresponding saline controls ($P<.05$).

[b]These values are significantly different from 100 ($P<.05$).

[c]These values are significantly different from corresponding 0 hour values ($P<.05$).

[d]This value is significantly different from that obtained after 6 hours of cessation of stimulation in presence of DMI ($P<.05$).

new steady state appears to be maintained by synthesis and retrieval of released norepinephrine. However, estimations of the ability of the terminals to synthesize norepinephrine are complicated by the slow rate of turnover of this amine in the resting state and by release, uptake, and metabolism of norepinephrine during stimulation. Therefore, the capacity of noradrenergic terminals to synthesize norepinephrine was studied by following the restoration of the tissue contents of this amine after periods of stimulation. Since norepinephrine presumably regulates its own synthesis by feedback inhibition of tyrosine hydroxylase the rate of recovery of norepinephrine would give an estimate of the capacity of terminals to synthesize norepinephrine. Decentralized preganglionic fibers were stimulated unilaterally for 3 hours at 10 hz and norepinephrine contents of tissues were determined immediately and at 2 and 6 hours after the cessation of stimulation.

The norepinephrine contents of ganglia were not altered by stimulation and during the post-stimulation period the ganglionic norepinephrine remained at control levels. In salivary glands stimulation reduced the norepinephrine content to 42% of control. Two hours after the cessation of stimulation the norepinephrine content increased to 65% of control and stabilized at this level for an additional 4 hours. The

post-stimulation changes in the norepinephrine content of nictitating membranes were similar to those of the salivary glands but were less pronounced (Table 19).

Recovery of norepinephrine contents could be due, in part, to the desmethylimipramine sensitive concentrating mechanism in noradrenergic terminals which retrieves norepinephrine from the circulating blood (Axelrod *et al.*, 1961; Strömblad and Nickerson, 1961; Kopin and Gordon, 1963). In order to test this possibility, and to accentuate the effects of stimulation on the norepinephrine contents of nictitating membranes, stimulation was carried out in the presence of desmethylimipramine. After 3 hours of stimulation the norepinephrine contents of salivary glands and nictitating membranes decreased to 25 and 58% of controls respectively. Two hours after cessation of stimulation the norepinephrine contents increased to 49% of control in salivary glands and to 78% of control in nictitating membranes; again they tended to plateau at these levels.

Since the initial rates of recovery of norepinephrine in salivary glands during the post-stimulation period in the absence of desmethylimipramine seemed to parallel the recovery of norepinephrine in the presence of this drug, it seems unlikely that uptake of norepinephrine from the circulation contributed to this recovery. The restoration of norepinephrine appeared to be due

Table 20. Formation of norepinephrine-^{14}C (NE-^{14}C) from dopamine-^{14}C six hours after the cessation of stimulation.

Tissue	Total radioactivity			NE-^{14}C			$\frac{\text{NE-}^{14}\text{C}}{\text{Total radioactivity}} \times 100$		
	NS	S	$\frac{S}{NS}$	NS	S	$\frac{S}{NS}$	NS	S	$\frac{S}{NS}$
Ganglia (dpm/10 mg)	1255± 633	3387± 1975	2.70[a]	267± 121	648± 349	2.43[a]	28.3± 6.9	23.5± 4.6	0.83
Salivary glands (dpm/g)	31232± 8796	23478± 7060	0.75	2783± 736	4529± 1410	1.63	10.06± 1.96	19.5± 1.36	1.94[a]
Nictitating membranes (dpm/memb)	12151± 4369	9987± 2630	0.82	974± 327	1300± 280	1.33	8.28± .90	13.6± 2.93	1.64[a]

The experiments were carried out in cats in which the preganglionic fibers were sectioned and the distal end stimulated intermittently (30 sec/min) at 10 hz for 3 hours. NS = nonstimulated; S = stimulated. Six hours after the cessation of stimulation, 2.5 μc of dopamine-^{14}C was infused through each common carotid artery for one-half hour. Values represent the mean ± 1 S.E. as determined from 5 separate experiments.

[a]These values are significantly different from 1 ($P<.05$).

primarily to synthesis of norepinephrine since the infusion of α-methyltyrosine, started immediately after the cessation of stimulation and continued for 6 hours, prevented the restoration of norepinephrine in salivary glands and nictitating membranes.

2. Conversion of dopamine-^{14}C to norepinephrine-^{14}C during the post-stimulation period

Following stimulation, the norepinephrine contents of salivary glands and nictitating membranes plateaued at levels that were markedly lower than controls (Figure 12; Table 19). It is not clear what factors limited the restoration of this amine. It has recently been demonstrated (Geffen *et al.*, 1969) that dopamine-β-hydroxylase is released into the venous effluent of spleen following stimulation of splenic nerves. Both storage vesicles and dopamine-β-hydroxylase, which are intimately associated, may be released together (Viveros *et al.*, 1969). Therefore, loss of this enzyme and/or the storage vesicles may be responsible for the failure of norepinephrine contents to recover completely following prolonged periods of stimulation. This possibility was tested by studying the conversion of dopamine-^{14}C to norepinephrine-^{14}C.

Dopamine-^{14}C was infused i.a. 6 hours after the cessation of stimulation as described in Methods. The results are summarized in Table 20. In nonstimulated

preparations the percentages of total radioactivity incorporated into norepinephrine-^{14}C in ganglia, salivary glands, and nictitating membranes were 28%, 10% and 8% respectively. These percentages of norepinephrine-^{14}C were greater than those found after the administration of tyrosine-^{14}C. This would be expected if the hydroxylation of tyrosine to DOPA was the limiting step in the synthesis of norepinephrine. There was no difference in the formation of norepinephrine-^{14}C in stimulated and nonstimulated ganglia. However, the formation of norepinephrine-^{14}C in salivary glands and nictitating membranes was significantly increased (1.9 and 1.6-fold respectively). These results suggest that the capacity of the noradrenergic terminals for storage and synthesis of norepinephrine from dopamine is not reduced following nerve stimulation. Indeed, the capacity of the stimulated terminals to accumulate norepinephrine-^{14}C from dopamine-^{14}C is enhanced.

3. Conversion of tyrosine-^{14}C to norepinephrine-^{14}C during and at various times after the cessation of stimulation

The results of studies on the conversion of dopamine-^{14}C to norepinephrine-^{14}C indicated that the absence of complete restoration of norepinephrine during post-stimulation period could not be due to a deficiency of dopamine-β-hydroxylase or storage sites

for norepinephrine. Therefore, the possibility that DOPA, and hence dopamine, could limit the complete recovery of norepinephrine was considered. Previous studies have demonstrated that electrical stimulation increased the formation of radioactive norepinephrine from tyrosine-^{14}C but not from DOPA-^{3}H (Sedvall and Kopin, 1967). This would suggest that the stimulus-induced increase in the formation of norepinephrine occurs at or before the tyrosine hydroxylation step and the formation of DOPA is the rate limiting step in the biosynthesis of norepinephrine. Conversion of tyrosine-^{14}C to norepinephrine-^{14}C was studied during the last one-half hour of a 3 hour stimulation period, immediately after, and at 2 and 6 hours after the cessation of the 3 hour stimulation period. The results are summarized in Tables 21-24 and for clarity the results of the studies with ganglia and salivary glands are graphically depicted in Figure 13.

During stimulation and at all times after the end of the stimulation period the formation of norepinephrine-^{14}C was similar in nonstimulated and stimulated ganglia. On the other hand, stimulation resulted in approximately 12- and 6-fold increases in the formation of norepinephrine -^{14}C in salivary glands and nictitating membranes respectively. Immediately after the cessation of stimulation the formation of radioactive amines in these

Table 21. Coversion of tyrosine-^{14}C to norepinephrine-^{14}C (NE-^{14}C) in superior cervical ganglia during and at various times after cessation of stimulation.

Tyrosine-^{14}C infused at	Total radioactivity (dpm/10 mg)			NE-^{14}C (dpm/10 mg)			$\frac{\text{NE-}^{14}\text{C}}{\text{Total radioactivity}} \times 100$		
	NS	S	$\frac{S}{NS}$	NS	S	$\frac{S}{NS}$	NS	S	$\frac{S}{NS}$
-1/2 hour poststimulation (3)	1,351± 805	1,748± 295	1.29	270± 191	269± 102	1.00	16.8± 2.7	14.3± 3.2	0.85
0 hour poststimulation (4)	2,151± 113	1,138± 324	0.53	465± 326	177± 52	0.38	16.2± 3.5	15.4± 1.1	0.95
2 hour poststimulation (3)	703± 91	907± 95	1.29	106± 18	120± 24	1.13	15.0± 1.5	13.0± 1.5	0.87
6 hour poststimulation (3)	2,848± 121	2,540± 149	0.89	223± 67	289± 156	1.30	8.7± 1.1	12.1± .8	1.39

The experiments were carried out in cats in which the preganglionic fibers were sectioned and the distal end stimulated intermittently (30 sec/min) at 10 hz for 3 hours. NS = nonstimulated; S = stimulated. 25 μc of tyrosine-^{14}C was infused through each common carotid artery for one-half hour during and at indicated periods after the cessation of stimulation. Values represent the mean ± 1 S.E. from the number of experiments indicated in parentheses.

Table 22. Conversion of tyrosine-^{14}C to norepinephrine-^{14}C (NE-^{14}C) in salivary glands during and at various times after cessation of stimulation.

Tyrosine-^{14}C infused at	Total radioactivity (dpm/g)		
	NS	S	$\frac{S}{NS}$
-1/2 hour poststimulation (3)	36,789± 8,663	74,215± 16,014	2.02[a]
0 hour poststimulation (4)	54,874± 16,117	78,681± 6,792	1.43
2 hour poststimulation (3)	36,032± 9,079	56,063± 24,377	1.56
6 hour poststimulation (3)	96,810± 44,833	59,951± 36,809	0.62

The experiments were carried out in cats in which the preganglionic fibers were sectioned and the distal end stimulated intermittently (30 sec/min) at 10 hz for 3 hours. NS = nonstimulated; S = stimulated. 25 μc of tyrosine-^{14}C was infused through each common carotid artery for one-half hour during and at indicated periods after the cessation of stimulation. Values represent the mean ± 1 S.E. from the number of experiments indicated in parentheses.

[a]These values are significantly different from 1 ($P<.05$).

Table 22 (Cont'd)

NE-^{14}C (dpm/g)			NE-^{14}C / Total radioactivity x 100		
NS	S	$\frac{S}{NS}$	NS	S	$\frac{S}{NS}$
55± 15	1,225± 153	22.27[a]	.15± .02	1.73± .18	11.53[a]
89± 31	874± 252	9.82	.16± .02	1.09± .29	6.81[a]
141± 62	625± 289	4.43	.36± .13	1.01± .38	2.80
119± 43	138± 80	1.16	.14± .04	.24± .03	1.71

Table 23. Conversion of tyrosine-^{14}C to norepinephrine-^{14}C (NE-^{14}C) in nictitating membranes during and at various times after cessation of stimulation.

Tyrosine-^{14}C infused at	Total radioactivity (dpm/membrane)		
	NS	S	$\frac{S}{NS}$
-1/2 hour poststimulation (3)	64,760± 11,772	39,080± 5,904	0.60
0 hour poststimulation (4)	69,600± 8,858	72,720± 10,243	1.04
2 hour poststimulation (3)	44,460± 4,140	54,320± 13,140	1.22
6 hour poststimulation (3)	103,880± 18,243	119,080± 22,722	1.15

The experiments were carried out in cats in which preganglionic fibers were sectioned and the distal end stimulated intermittently (30 sec/min) at 10 hz for 3 hours. NS = nonstimulated; S = stimulated. 25 μc of tyrosine-^{14}C was infused through each common carotid artery for one-half hour during and at indicated periods after the cessation of stimulation. Values represent the mean ± 1 S.E. from the number of experiments indicated in parentheses.

[a]These values are significantly different from 1 ($P<.05$).

Table 23 (Cont'd)

NE-^{14}C (dpm/membrane)			NE-^{14}C / Total radioactivity x 100		
NS	S	$\frac{S}{NS}$	NS	S	$\frac{S}{NS}$
61± 13	196± 14	3.21	0.09± 0	0.50± .08	5.56[a]
64± 14	207± 44	3.23	0.09± 0	0.29± .04	3.22[a]
56± 34	122± 82	2.18[a]	0.09± .04	0.19± .09	2.11
73± 12	91± 20	1.25	0.07± 0	0.08± 0	1.14

Table 24. Effect of desmethylimipramine (DMI) on the conversion of i.a. administered tyrosine-^{14}C to norepinephrine-^{14}C (NE-^{14}C) six hours after the cessation of stimulation.

Tissue	Total radioactivity		
	NS	S	$\frac{S}{NS}$
Ganglia (dpm/10 mg)	3,795± 2,128	2,280± 402	0.60
Salivary glands (dpm/g)	22,864± 5,908	57,589± 25,466	2.52
Nictitating membranes (dpm/membrane)	70,320± 20,911	42,840± 8,750	0.61

The experiments were carried out in cats in which preganglionic fibers were sectioned and the distal end stimulated intermittently (30 sec/min) at 10 hz for 3 hours. NS = nonstimulated; S = stimulated. Two-thirds of the dose of DMI (3 mg/kg) was injected i.v. concomitant with the start of stimulation and another one-third of the dose 2 hours later. 25 μc of tyrosine-^{14}C was infused through each common carotid artery for one-half hour 6 hours after the cessation of stimulation. Values represent the mean ± 1 S.E. as determined from three separate experiments.

Table 24 (Cont'd)

$NE-^{14}C$			$\frac{NE-^{14}C}{\text{Total radioactivity}} \times 100$		
NS	S	$\frac{S}{NS}$	NS	S	$\frac{S}{NS}$
599± 240	344± 131	0.60	18.81± 2.72	14.21± 4.26	0.76
186± 138	983± 848	5.28	0.64± 0.37	1.08± 0.75	1.70
218± 172	65± 26	0.30	0.24± 0.16	0.14± 0.05	0.60

Figure 13. Norepinephrine content and formation of norepinephrine-^{14}C (NE-^{14}C) from tyrosine-^{14}C following stimulation and during poststimulation periods.

The experiments were carried out in cats in which the preganglionic fibers were sectioned and the distal end stimulated intermittently (30 sec/min) at 10 hz for 1 hour (solid bar) or 3 hours (open bar). Twenty-five μc of tyrosine-^{14}C was infused through each common carotid artery for one-half hour during the last one-half hour of a 3 hour stimulation period, immediately after and at 2 and 6 hours after the cessation of the 3 hour stimulation period. In the upper panel the NE contents of ganglia and salivary glands is expressed as percent of nonstimulated controls. Each point represents the mean from 3 to 5 experiments. Vertical bars at each point indicates the ± 1 S.E. of the mean. The height of each bar in the lower panel represents the mean dpm of NE-^{14}C expressed as the difference between the stimulated and nonstimulated tissues. Vertical lines projected on each bar represents the ± 1 S.E. as determined from 3 to 4 experiments.

*Values are significantly different from 100% ($P<.05$).

**Values are significantly different from 0 ($P<.05$).

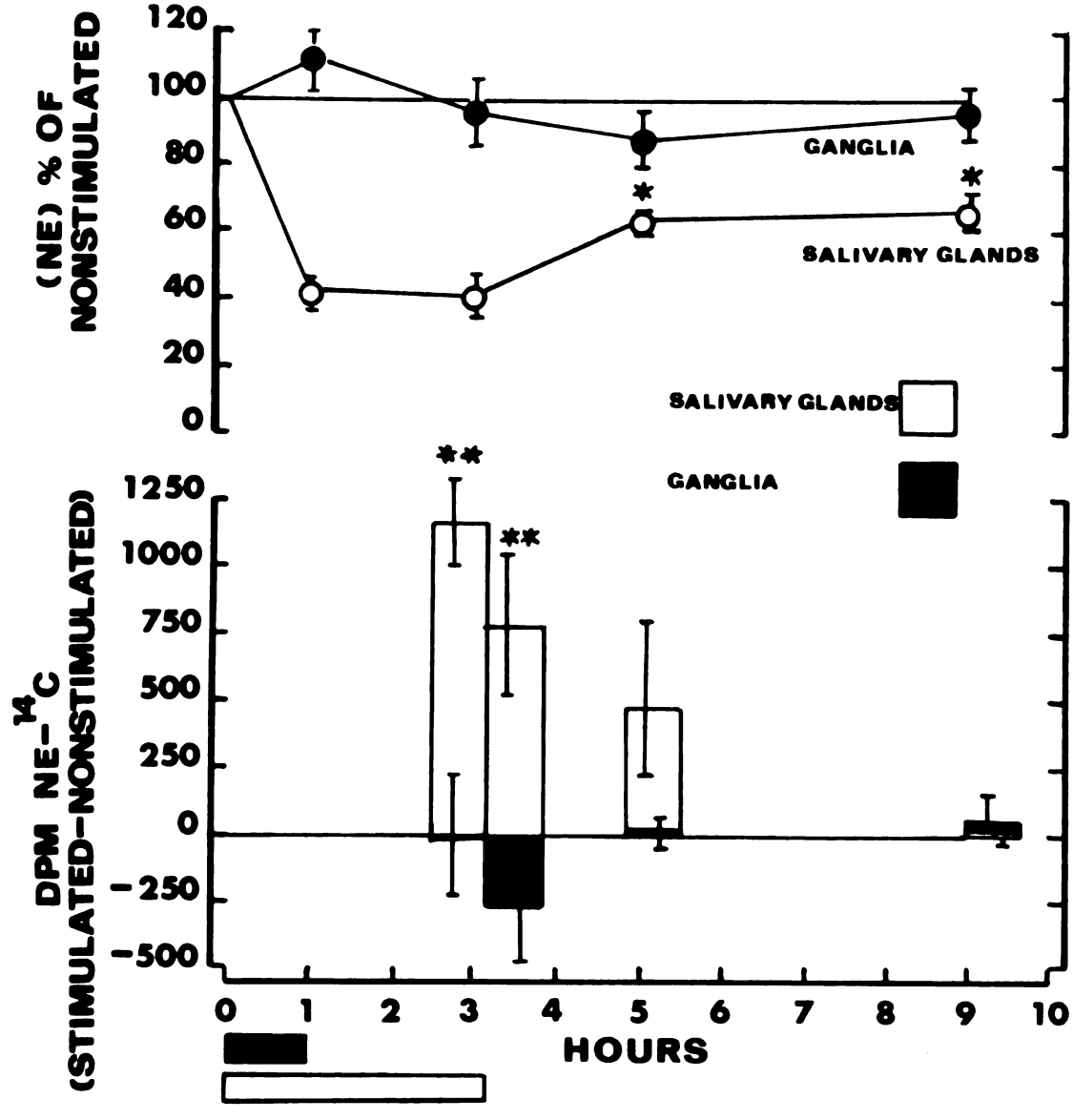

Figure 13. Norepinephrine content and formation of norepinephrine-^{14}C (NE-^{14}C) from tyrosine-^{14}C following stimulation and during poststimulation periods.

tissues continued at an accelerated rate. Two hours later the formation of norepinephrine-^{14}C was still elevated but by 6 hours after the cessation of stimulation the rate of norepinephrine-^{14}C formation was the same in stimulated and nonstimulated salivary glands and nictitating membranes. The time course of the increased formation of norepinephrine-^{14}C did not parallel the reduced endogenous norepinephrine. That is, as the endogenous norepinephrine contents began to increase the formation of norepinephrine-^{14}C progressively decreased. Six hours after the cessation of stimulation when the endogenous norepinephrine contents were well below (35%) control, the synthesis of norepinephrine had returned to control levels. Similar results were obtained in a series of experiments where desmethylimipramine was administered to accentuate the stimulus-induced depletion of norepinephrine in nerve terminals (see Table 19). That is, 6 hours after the cessation of stimulation, endogenous norepinephrine contents plateaued at 35% below control (Figure 12), but synthesis returned to control levels. These results suggest that only part of the norepinephrine stores in noradrenergic terminals participate in regulating the norepinephrine synthesis in nerve terminals.

DISCUSSION

A. Regulation of Norepinephrine Content in Neuronal Cell Bodies and Terminals During Stimulation

A series of events take place in the terminals of noradrenergic neurons following electrical stimulation. Stored norepinephrine is released (Brown and Gillespie, 1957), synthesis of norepinephrine is accelerated (Weiner and Rabadjija, 1968), and released norepinephrine is recaptured (Iversen, 1967). Although the dynamics of norepinephrine at nerve terminals have been studied extensively little is known about the properties of this amine in the cell body.

Small amounts of norepinephrine may be released from autonomic ganglia following anti- or orthodromic electrical stimulation (Bülbring, 1944; Reinert, 1963), but evidence for a modulating action of norepinephrine on ganglionic transmission is weak (Costa *et al.*, 1961). Some drugs such as reserpine and α-methyltyrosine (Fischer and Snyder, 1965; Reinert, 1963; Brodie *et al.*, 1966), deplete ganglia of norepinephrine whereas other drugs are without effect (e.g., tyramine; Fischer and Snyder, 1965). Continuous electrical stimulation does not alter the norepinephrine content of cat superior cervical

ganglia (Reinert, 1963). Similar results were obtained in the present study; that is, α-methyltyrosine produced a marked depletion of norepinephrine in the superior cervical ganglia (Figure 5; Table 2) whereas 1 or 3 hours of preganglionic or postganglionic electrical stimulation (Table 4; Table 5) at low or high frequencies (Table 5) did not.

Although functions of ganglionic norepinephrine are unknown the properties of this amine in cell bodies are quite distinct from those in the terminals. For example, the rate of turnover of norepinephrine is much greater in ganglia than in neuroeffector organs (Brodie *et al.*, 1966). This is evidenced in the present study by the fact that α-methyltyrosine caused a rapid decline in the norepinephrine content in cell bodies but not in tissues that contain terminals (Table 2; Figure 5). Chronic decentralization is reported to reduce the turnover rate and increase the content of norepinephrine in ganglia (Kirpekar *et al.*, 1962; Fischer and Snyder, 1965), but in the present study acute decentralization (Table 2) or 3 hours of preganglionic stimulation (Figure 7) did not alter the α-methyltyrosine-induced decline of norepinephrine.

It is likely that synthesis of norepinephrine in the ganglia is proceeding at a maximal rate--a rate determined by innate activity of noradrenergic neurons. The activity of tyrosine hydroxylase is much higher in

cell bodies and axons than it is in terminals and cannot be altered by short term changes in neuronal activity (Roth *et al.*, 1967; Sedvall and Kopin, 1967). In the present study, cycloheximide, an inhibitor of protein synthesis, increased the ganglionic-content of norepinephrine (Table 12). This is contrary to what would be expected if the high turnover rates of norepinephrine were due to relatively high turnover rates of tyrosine hydroxylase. The accumulation of norepinephrine in cell bodies in presence of cycloheximide could not have been due to block of axonal transport of norepinephrine because inhibition of protein synthesis does not effect the rates of axonal transport (Peterson *et al.*, 1967). Furthermore, an increase in norepinephrine concentration was also obtained in the noradrenergic terminals of nictitating membranes (Table 12). The effects of cycloheximide on the tissue contents of norepinephrine probably result from some nonspecific, and as yet unknown, mechanism which is secondary to inhibition of protein synthesis (Yeh and Shils, 1969). Indeed, after administration of tyrosine-^{14}C the total radioactivity in the ganglia, presumably the non-metabolized amino acid, was much higher in the presence than in the absence of cycloheximide (compare Tables 10 and 14). No systematic studies were conducted on the effect of inhibition of protein synthesis on the turnover of ganglionic norepinephrine. The possibility cannot be excluded, however,

that elevated concentration of norepinephrine in the ganglia following inhibition of protein synthesis may have resulted from a decreased turnover of this amine.

Desmethylimipramine, in the presence or absence of stimulation, did not alter the content of ganglionic norepinephrine (Table 8; Figure 6) and did not affect the uptake of norepinephrine-^{3}H in the stimulated ganglia (Table 9). In addition, desmethylimipramine did not modify the effect of α-methyltyrosine. That is, the decline of ganglionic norepinephrine after α-methyltyrosine was the same in the presence or absence of desmethylimipramine; therefore, reuptake does not appear to play a major role in the maintenance of this amine in cell bodies. A similar conclusion was reached by Fischer and Snyder (1965) as a result of their studies with cocaine.

What is the fate of norepinephrine in ganglia? Dahlström and Häggendal (1970) have reported that norepinephrine, supposedly in storage granules, is transported down the axon. In the present studies some of the norepinephrine in the ganglia appeared to be transported somatofugally since a ligature around the post-synaptic nerve bundle adjacent to the ganglia partially prevented the α-methyltyrosine-induced decline of norepinephrine. Nevertheless, the norepinephrine content of the tied ganglia was reduced. This might be

due to incomplete blockade of axonal transport or sparing of some postganglionic fibers by the ligature, replacement of the norepinephrine by a metabolite of α-methyltyrosine (i.e., α-methylnorepinephrine), metabolism of norepinephrine by monoamine oxidase, or release of the norepinephrine into the blood. The latter possibility was not examined, but metabolism by monoamine oxidase does not appear to be an important factor. Although this enzyme is found in the superior cervical ganglion of the cat (Giacobini and Kerpel-Fronius, 1970) it plays little role in the metabolism of norepinephrine in this species (Spector *et al.*, 1963). In the present study, pretreatment with a monoamine oxidase inhibitor did not prevent the reduction of the norepinephrine content in the ganglia following α-methyltyrosine administration. The increase in the ganglionic norepinephrine that was obtained after ligating the postganglionic fibers was the same in the presence or absence of a monoamine oxidase inhibitor (Table 16). In addition, replacement of norepinephrine by α-methylnorepinephrine did not appear to account for the decline of norepinephrine stores since little of this methylamine could be detected in the tissues after infusions of α-methyltyrosine-^{3}H (Table 17; see also Van Orden *et al.*, 1970b).

One hour of preganglionic stimulation at 10 hz reduced the norepinephrine content of nerve terminals

in the salivary glands. This reduced concentration of norepinephrine was maintained when stimulation was continued for 3 hours (Figure 9). Although, α-methyltyrosine did not significantly alter the norepinephrine content in resting nerve terminals it increased the stimulus-induced reduction of norepinephrine. The lack of a significant effect of α-methyltyrosine in the resting state would be expected if the turnover of norepinephrine in the terminals is slow (Figure 5; see also Fischer and Snyder, 1965). On the other hand, increased utilization of norepinephrine at the nerve terminals following stimulation presumably results in a decreased product inhibition of tyrosine hydroxylase and consequently an acceleration of norepinephrine synthesis (Weiner and Rabadjija, 1968) which is prevented by α-methyltyrosine. Similar effects were obtained with desmethylimipramine; that is, desmethylimipramine alone or in combination with α-methyltyrosine did not significantly alter the norepinephrine content in resting nerve terminals (Table 3; Figure 6), but increased the stimulus-induced reduction of norepinephrine and decreased the uptake of norepinephrine-^{3}H in stimulated terminals (Figure 7; Table 9). In salivary glands a combination of α-methyltyrosine and desmethylimipramine almost totally depleted norepinephrine as early as 1 hour after stimulation, indicating that almost all the norepinephrine in this tissue can be

mobilized for release. This suggests that during periods of increased nerve activity both synthesis and reuptake are operating in an effort to maintain the norepinephrine contents. These two mechanisms, however, cannot maintain the norepinephrine concentrations at the normal values and a new steady state concentration of norepinephrine is reached if stimulation is continued for 3 hours (Figure 9). Brown (1960) obtained a similar rapid decline in the norepinephrine contents of spleen following stimulation of the splenic nerves; the reduced norepinephrine concentration was maintained over long periods of stimulation. Indeed, in the absence of synthesis and uptake all of the amine could be conceivably depleted in less than 15 min after the start of stimulation at 4 hz (Haefely *et al.*, 1965).

The relative importance of the processes of synthesis and reuptake in maintaining norepinephrine stores is, however, still controversial. For example, in the nerve terminals, either uptake of norepinephrine (Hedqvist and Stjärne, 1969) or synthesis of this amine (Malmfors, 1964) have been proposed to be of prime importance during periods of increased nerve activity. The relative roles of synthesis and reuptake of norepinephrine appear to be dependent on the frequency of stimulation (Table 5). At low frequencies only synthesis, and at high frequencies both synthesis and reuptake of norepinephrine contribute to the maintenance of norepinephrine in the

nerve terminals; similar results have been reported by Bhagat and Friedman (1969). In addition, the magnitude of stimulus-induced reduction in the norepinephrine content of salivary glands in the presence of α-methyltyrosine is similar at low and high frequencies of stimulation (Table 5). That is, at a frequency of 2 hz, α-methyltyrosine reduced the norepinephrine content by 24% (from 81% of control to 57% of control) and at 10 hz by 30% (from 42% of control to 12% of control). The extent of stimulus-induced reduction in the norepinephrine contents of salivary glands in presence of α-methyltyrosine was similar at 2 hz and 10 hz even in combination with desmethylimipramine. This suggests that increased nerve activity triggers the synthesis of norepinephrine at a rate which is independent of the frequency and the initial concentration of norepinephrine. Reuptake of released norepinephrine becomes of major importance for the maintenance of norepinephrine for transmitter function only at higher frequencies of stimulation.

Qualitatively, the results obtained with studies of the nerve terminals in nictitating membranes are similar to those obtained with the salivary glands. Quantitative discrepancies between these two tissues are apparently due to the less pronounced effects of stimulation on the norepinephrine content of nictitating

membranes and are further complicated by crude dissection of the membranes. Functional and morphological characteristics of the smooth muscle of the membranes may preclude a large release of transmitter following stimulation. Nictitating membranes have a dense noradrenergic innervation and the distance between nerve endings and smooth muscle is less than 300 Å (Van Orden *et al.*, 1967). Such an arrangement would favor an efficient uptake process and thus conserve transmitter. Another factor might be a decreased blood flow during stimulation and consequently a decreased loss of released amine into the circulation. Blood flow of skeletal muscle is reduced 2 to 5-fold following stimulation at 5 to 12 hz whereas norepinephrine is reduced 30% and 53% respectively (Kernell and Sedvall, 1964); even stimulation at 0.5 hz results in an increase in vascular resistance (Fredholm and Sedvall, 1966). The quantitative differences in the disposition of norepinephrine in the terminals of nictitating membranes and salivary glands might also be explained if a substantial amount of this amine in the membranes is extraneuronal (Draskóczy and Trendelenburg, 1970) and thus is not affected by stimulation.

Studies with radioactive tyrosine confirmed that the rate of synthesis of norepinephrine in cell bodies was faster than in the terminals. The results with the

radioactive isotopes also indicated that synthesis of norepinephrine in ganglia proceeds independent of changes in afferent input. That is, preganglionic stimulation did not alter the rate of synthesis of norepinephrine in the cell bodies but it markedly increased the rate of formation of norepinephrine in salivary glands and nictitating membranes (Tables 10 and 11).

The total radioactivity in the stimulated and non-stimulated ganglia was the same, but in salivary glands the total radioactivity on the stimulated side was doubled. The converse was true in the nictitating membranes where total radioactivity was reduced in the stimulated side. Since catecholamines represent only a small percentage of the total radioactivity in the terminals (0.1-2%) it was assumed that most of this radioactivity represents tyrosine-^{14}C. The increased total radioactivity in the stimulated salivary glands may have resulted from compensatory vasodilation and increased blood flow (Bhoola *et al.*, 1965), increased transport of tyrosine into the nerve terminals, or increased incorporation of tyrosine into protein in the salivary gland. It was quite evident, however, that the increase in norepinephrine formation was greater than the increase in total radioactivity (total radioactivity increased 2 fold while the formation of norepinephrine increased 22 fold). The reduced total radioactivity in stimulated nictitating membranes may have resulted from

vasoconstriction and decreased blood flow. Despite this, however, there was a marked increase in the conversion of tyrosine-^{14}C to norepinephrine-^{14}C. The effects with i.a. infusions of tyrosine-^{14}C were essentially the same as those obtained following i.v. infusions of this amino acid. That is, the formation of norepinephrine-^{14}C was markedly increased in stimulated terminals (salivary glands and nictitating membranes) but not in the cell bodies.

It has been suggested that rapid alterations of norepinephrine synthesis in nerve terminals accompanying nerve stimulation are obtained without the necessity for an increase in the enzyme protein (Sedvall and Kopin, 1967a). In the present studies cycloheximide decreased the formation of norepinephrine-^{14}C from tyrosine-^{14}C (Table 14). Although these results do not support the contention that immediate acceleration of synthesis of norepinephrine during stimulation is due to the mechanism of product inhibition and not due to increased protein synthesis, the effects of inhibition of protein synthesis in present studies are equivocal. Cycloheximide does not alter the stimulus-induced decline in the norepinephrine content of nerve terminals (Table 13). If the synthesis of the amine was blocked, a greater reduction in the norepinephrine content would be expected. Cycloheximide could reduce the transport and incorporation of tyrosine into various metabolic pools in the tissues.

Indeed the total radioactivity was much higher in all tissues in the presence of cycloheximide (compare Tables 10 and 14). The stimulus-induced synthesis of norepinephrine, however, was still elevated in the nerve terminals although to a lesser extent than it was in the absence of an inhibitor of protein synthesis. It is unlikely that a reduction in the transmitter synthesis in the presence of cycloheximide reflects a reduction in the enzyme proteins. Puromycin inhibits the stimulus-induced increase in the synthesis of norepinephrine (Weiner and Rabadjija,1968) at a time following stimulation when the amount of tyrosine hydroxylase is not altered (Weiner, 1970). Furthermore, when the salivary glands were stimulated for 3 hours and the slices of this tissue incubated in presence of tyrosine-^{14}C the formation of norepinephrine-^{14}C increased approximately 5 fold as compared to nonstimulated glands. This increase was markedly reduced in the presence of $10^{-5}M$ norepinephrine and almost completely inhibited in the presence of $10^{-4}M$ norepinephrine (Chieuh *et al.*, 1971).

Many efforts have been made to correlate the norepinephrine content and physiological responses of noradrenergic effector tissues (Iversen, 1967). Generally, the norepinephrine content of nerve terminals can be markedly reduced without causing a comparable reduction in the responses of tissues to drugs or

stimulation. Although the responses of salivary glands and pupils following stimulation of the sympathetic trunk were not quantified in the present study some attempt was made to quantify the responses of the nictitating membranes to nerve stimulation. In agreement with Thoenen *et al.* (1966) no consistent relationship between the amount of norepinephrine and the degree of contraction of the membranes was obtained (Table 18; Figures 10 and 11). The results, however, are complicated by the fact that long periods of stimulation may injure nerve fibers or cause ischemia. The lack of functional correlation with tissue contents of norepinephrine is not a characteristic of all noradrenergic tissues. A good correlation was obtained between the norepinephrine content and contraction of splenic nerve following stimulation and the administration of α-methyltyrosine (Thoenen *et al.*, 1966). Factors other than the absolute amounts of transmitter are probably important determinants of the pharmacology of noradrenergic tissues; for example, the availability of newly synthesized norepinephrine (Kopin *et al.*, 1968) and the morphological organization of noradrenergic synapse. The latter may vary from one tissue to another (Trendelenburg, 1969).

B. Regulation of Norepinephrine Content in the Neuronal Cell Bodies and Terminals After Stimulation

One of the means of studying noradrenergic mechanism has been to investigate the dynamics of neuronal norepinephrine during and after periods of increased nerve activity. Clearly, the amount of norepinephrine in the neuron during stimulation depends upon a number of factors: release, synthesis, uptake of released norepinephrine, catabolism, and overflow into the circulation. The mechanism of synthesis, uptake, and axonal transport cannot maintain the normal norepinephrine concentration in the nerve terminals in the presence of high frequency stimulation so that the tissue is rapidly depleted of this amine. It is not clear, what factors control the replenishment of norepinephrine after its depletion and in what manner the changes in stimulation-induced nerve activity alter the dynamics of neuronal norepinephrine once the source of the increased nerve activity is eliminated.

In the cell bodies, although acute changes in the sympathetic activity do not alter the norepinephrine content chronic increases in functional demands increase tyrosine hydroxylase in the cell bodies of both peripheral and central nervous systems (Mueller *et al.*, 1969a, b; Thoenen *et al.*, 1969; Thoenen, 1970). However, it takes 12 to 24 hours before this increase becomes apparent.

In the present study neither the content of norepinephrine nor the formation of norepinephrine-^{14}C from tyrosine-^{14}C or dopamine-^{14}C was altered in the cell bodies during the 6 hour period following the cessation of 3 hours of stimulation (Figure 13; Table 21). This suggests that the changes in dynamics of norepinephrine in the cell bodies are not apparent until many hours after stimulation, and when they do occur they are a consequence of adaptation to long term functional demands.

In the terminals, the norepinephrine content increases rapidly after a stimulus-induced depletion (Figure 12; Table 19). This increase is not due to the uptake of circulating norepinephrine since similar results are obtained in the presence of desmethylimipramine. The increase appears to be largely due to synthesis because it can be prevented by administration of α-methyltyrosine (Figure 12). The norepinephrine content, however, does not reach normal levels following stimulation. Instead, it appears to plateau within 6 hours of cessation of stimulation at a level that is significantly below control. Following stimulation-induced depletion of norepinephrine, synthesis of norepinephrine proceeds at an accelerated rate for 2 hours, but returns to control values 4 hours later when the endogenous norepinephrine contents are still below control (Figure 13). These results are contrary to those reported by other workers.

Fredholm and Sedvall (1966) noted a complete restoration of norepinephrine contents of rat salivary glands in less than 3 hours after cessation of stimulation; however, they stimulated the sympathetic trunk for 30 min at 5 hz. The rate of synthesis of norepinephrine during the post-stimulation period obtained by Fredholm and Sedvall was 0.5 µg/g/hr and is about 4 times that reported by Costa (1969) in resting rat salivary glands. During the first 2 hours after cessation of stimulation the norepinephrine content in cat salivary glands increased at a rate of 0.10 µg/g/hr (Table 19; Figure 12). Thus, during the post-stimulation periods norepinephrine stores in peripheral tissues are restored largely by synthesis which proceeds at a rate that far exceeds the turnover rates of norepinephrine in the resting state. The increased synthesis of norepinephrine during the post-stimulation period is most likely due to the lack of end-product inhibition since the increase in the synthesis of the amine could be markedly reduced or prevented when the slices of stimulated salivary glands were incubated with norepinephrine (Chieuh *et al.*, 1971). In addition, the rate of restoration of norepinephrine does not appear to be dependent on the extent of the depletion of the amine. The rates of restoration of norepinephrine in the salivary glands after a depletion to 45% of control by stimulation alone

or to 25% of control by stimulation in the presence of desmethylimipramine appear to be similar (Figure 12). In the presence of desmethylimipramine the amine contents also tended to plateau below control levels and the formation of norepinephrine-^{14}C from tyrosine-^{14}C returned to control values before endogenous stores of norepinephrine were restored (Table 24).

Several possibilities may be considered to explain the lack of complete restoration of norepinephrine in the nerve terminals following stimulus-induced depletion. In the nerve endings, norepinephrine is stored in characteristic storage vesicles that are intimately associated with dopamine-β-hydroxylase (Van Orden *et al.*, 1966; Hökfelt, 1969; Viveros *et al.*, 1969). It has been reported that dopamine-β-hydroxylase and storage vesicles are released from the spleen and adrenal glands during stimulation (Geffen *et al.*, 1969; Viveros *et al.*, 1969; Gewirtz and Kopin, 1970), but the release of dopamine-β-hydroxylase from other tissues remains controversial (Stjärne *et al.*, 1970). If dopamine-β-hydroxylase is lost from salivary glands, then even if there is accelerated synthesis of dopamine from tyrosine, the endogenous norepinephrine content will remain subnormal. In the present study, however, a deficiency of dopamine-β-hydroxylase does not appear to prevent the restoration of norepinephrine content in

the terminals since the formation of norepinephrine-^{14}C from dopamine-^{14}C was increased 2-fold in the terminals (Table 20). These results, however, do not preclude some loss of the enzyme and storage vesicles. The increase in the formation of norepinephrine-^{14}C from radioactive dopamine may result from an increase in the membrane transport of dopamine or from the induction of dopamine-β-hydroxylase. Kvetňanský *et al.* (1971) reported an increase in the amount of dopamine-β-hydroxylase in the adrenal glands 6 hours after a stress-induced increase in the sympathetic discharge; this increase was blocked by actinomycin D and cycloheximide.

Only part of the total norepinephrine stores in the nerve endings may exert a product-inhibition of tyrosine hydroxylase. Multiple "compartments" of norepinephrine in the nerve endings have been postulated (Kopin, 1966) and only a "strategically" located compartment is thought to exert feedback inhibition of tyrosine hydroxylase (Weiner and Rabadjija, 1968). Since most of the norepinephrine contents of salivary glands can be mobilized for release (Figure 9), and only part of the normally occurring norepinephrine contents are restored by synthesis (Figure 12) more than one store for norepinephrine probably exists in the nerve endings. Norepinephrine is presumably mobilized for release from all stores in the salivary glands during stimulation.

The release of the amine frees tyrosine hydroxylase from product inhibition and the norepinephrine synthesis is accelerated (Figure 13; Tables 22 and 23). The synthesis of norepinephrine returns to control levels when the strategic compartment of norepinephrine is restored (Figure 13). Since the residual storage vesicles which have released their amine content might still be empty, they can elicit an increased conversion of dopamine-^{14}C to norepinephrine-^{14}C (Table 20). This suggests that formation of DOPA is limiting the restoration of norepinephrine contents in the nerve endings, or that the tissue contents of norepinephrine are not exclusively regulated by the mechanism of feed-back inhibition.

The results of this study confirm previous reports which indicate that an increase in tyrosine hydroxylase in terminals cannot be detected until many hours (at least more than 6) after a period of increased sympathetic discharge (Dairman and Udenfriend, 1970). Furthermore, induced changes in the tyrosine hydroxylase appear in the cell body before they are detected in terminals indicating that inductive changes in enzyme protein are initiated in the cell bodies (Axelrod *et al.*, 1970).

C. The Noradrenergic Neuron: A Single Functional Unit

The results of degeneration studies suggest that long and ramified neuronal processes are dependent upon the soma for their integrity. The regulation of

adjustment of noradrenergic mechanism in cell bodies and terminals to increased functional demands is not well understood but both chemical and physical processes may be involved. It would appear that cell bodies do not contribute significantly to the dynamics of norepinephrine in terminals during acute stimulation. The total norepinephrine content of cell bodies of noradrenergic neurons in one ganglion is approximately 90 nanograms whereas the terminals of these neurons in the salivary glands and nictitating membranes contain approximately 1000 nanograms. Only 17-19 nanograms of ganglionic norepinephrine were lost through axonal transport in 3 hours (Table 16). The amount of stimulus-induced release of norepinephrine from salivary glands and nictitating membranes is not known but the amount of amine released from spleen has been reported to be 14.68 and 12.19 ng/DNA-unit/min at 5 and 10 hz respectively (Hedqvist and Stjärne, 1969). In the present studies, if the rate of the initial rapid decline in the norepinephrine content of salivary glands following 1 hour of stimulation at 10 hz is taken as an index of release, the estimate of the rate of synthesis would approximate that obtained by Hedqvist and Stjärne (1969) (Figure 9; Table 7). Thus, during periods of acute stimulation the cell body could not contribute significantly to the norepinephrine content

in the terminal. The same conclusion was reached as a result of experiments in the cat spleen (Geffen and Rush, 1968).

Although no function can be ascribed to norepinephrine in ganglia it is known that synthesis of this amine in cell bodies proceeds at a rapid rate independent of neuronal activity (Table 5). Roth *et al.* (1967) have reported similar results in bovine splenic nerve. In cell bodies, norepinephrine forms an aggregate with protein (Lubinska, 1964; Dahlström and Häggendal, 1970). These aggregates slowly mature, as evidenced by differential staining characteristics during their somatofugal transport and concentrate in the nerve terminals where they are referred to as storage granules. Once in the terminals the granules are available for release, storage, uptake and synthesis of norepinephrine. In the terminals the amine is released, tyrosine hydroxylase is freed of feedback inhibition, and synthesis of norepinephrine is thereby enhanced. Since norepinephrine does not appear to be released from cell bodies or axons, a similar control of synthesis is probably not necessary in these parts of the neuron. The amine may be present as a biological redundancy, only because all the enzymes and substrates necessary for its synthesis are available (Fischer and Snyder, 1965; Laduron and Belpaire, 1968). The turnover rate of

tyrosine hydroxylase (Mueller *et al.*, 1969a, b) and the half life of norepinephrine storage granules (Dahlström and Häggendal, 1970) has been estimated to be 8 and 22 days respectively. Accordingly, the terminals can maintain their ability to synthesize, recapture, and store norepinephrine for relatively long periods of time. Thus, in terminals, unlike in the cell bodies, minute to minute regulation of the norepinephrine content is accomplished by synthesis and by retrieval of released norepinephrine. All of the normally occurring tissue contents of norepinephrine, however, may not exert a product inhibition and the transmitter may be located in more than one morphological entity. Indeed, newly synthesized norepinephrine is preferentially released following stimulation of splenic nerves (Kopin *et al.*, 1968).

In addition to the immediate changes in the end-product inhibition of tyrosine hydroxylase (Weiner and Rabadjija, 1968) the neuronal norepinephrine contents can be modulated by gradual alterations in the amount of tyrosine hydroxylase and dopamine-β- hydroxylase (Thoenen, 1970; Kvetňanský *et al.*, 1971) that are induced by prolonged sympathetic discharge. The induction of tyrosine hydroxylase in adrenal glands and ganglia following a drug-induced chronic increase in afferent input takes at least 12 hours (Thoenen *et al.*, 1969; Mueller *et al.*, 1969a, b). Induction of tyrosine hydroxylase in the cell bodies of hind brain following

a stress-induced increase in the afferent input is obtained within 24 hours of the stress (Thoenen, 1970). The amount of tyrosine hydroxylase also increases in the terminals following compensatory increases in the sympathetic activity (Dairman and Udenfriend, 1970), but the inductive changes in the enzyme protein are initiated in the cell body and precede the enzymic alterations in the terminals (Axelrod, *et al.*, 1970).

Thus, the neuronal processes of a noradrenergic neuron might be considered in a dynamic state in which the nerve terminals are reformed and refashioned to suit the physiological demands whereas the soma appears to function as a source of enzymes and assembly of the storage mechanism for the transmitter. The short term regulation of norepinephrine concentrations in terminals and cell bodies are maintained independent of one another. Accordingly, the dynamics of norepinephrine in various parts of the neuron are different. These differences must be considered when interpreting the results of studies involving norepinephrine turnover, metabolism and uptake, etc. in tissues such as brain, gut, vas deferens, uterus, etc., that contain both cell bodies and terminals.

SUMMARY AND CONCLUSIONS

The purpose of the present study was to investigate the factors that regulate the norepinephrine content in superior cervical ganglia, which represent cell bodies, and in submaxillary salivary glands and nictitating membranes, which contain nerve terminals of post-ganglionic sympathetic neurons, during stimulation and post-stimulation periods.

In decentralized, nonstimulated noradrenergic neurons, 3 hours of α-methyltyrosine infusion reduced the norepinephrine content in the cell bodies but not in the terminals. The α-methyltyrosine-induced depletion of norepinephrine in cell bodies was retarded if a ligature was tied around the axon. Desmethyl-imipramine did not alter the norepinephrine content of cell bodies or terminals.

Low or high frequencies of stimulation, alone or in combination with desmethylimipramine or α-methyl-tyrosine, did not alter the norepinephrine content in the cell bodies. On the other hand, 1 hour of stimulation alone at 10 hz partially depleted norepinephrine in the terminals. The reduced concentration of norepinephrine was maintained if stimulation was continued for 3 hours.

The administration of α-methyltyrosine enhanced the stimulus-induced depletion at both low and high frequencies, whereas desmethylimipramine increased the depletion of norepinephrine only at high frequences. The combination of α-methyltyrosine and desmethylimipramine further enhanced the norepinephrine depletion at high frequencies of stimulation. The α-methyltyrosine-induced depletion did not result from the replacement of norepinephrine by α-methylnorepinephrine.

Preganglionic stimulation at 10 hz had little effect upon the conversion of tyrosine-^{14}C to norepinephrine-^{14}C in the cell bodies but increased the formation of norepinephrine-^{14}C in the terminals. α-Methyltyrosine reduced the synthesis of norepinephrine-^{14}C in all tissues.

Decentralized preganglionic fibers were stimulated at 10 hz for 3 hours and the tissue contents of norepinephrine were determined immediately after and at 2 and 6 hours after the cessation of stimulation. In the cell bodies neither endogenous norepinephrine content nor the formation of norepinephrine-^{14}C from tyrosine-^{14}C was altered. In the terminals, stimulation alone reduced the norepinephrine content; the concentrations of norepinephrine increased rapidly within 2 hours after cessation of stimulation but then plateaued and during the next 4 hours did not return to control values. Partial restoration of norepinephrine

was prevented by α-methyltyrosine but not by desmethylimipramine. The rate of formation of norepinephrine-^{14}C was accelerated during the 30 minute period immediately after cessation of stimulation but thereafter decreased progressively as the endogenous norepinephrine concentrations increased. At 6 hours after cessation of stimulation, when the endogenous concentrations of norepinephrine were below control, the rate of conversion of tyrosine-^{14}C to norepinephrine-^{14}C was not different from nonstimulated controls whereas the conversion of dopamine-^{14}C to norepinephrine-^{14}C was increased 2-fold.

The results suggest that in cell bodies synthesis of norepinephrine proceeds at a rapid rate that is independent of nerve activity and concentrations of norepinephrine are maintained only by synthesis. In terminals, synthesis of norepinephrine proceeds at a slow rate in the absence of nerve activity; with low frequencies of stimulation concentrations of norepinephrine are partially maintained by synthesis and at higher frequencies by both synthesis and reuptake.

All immediate requirements for the transmission process are fulfilled locally at the terminals of noradrenergic nerves. When the norepinephrine contents of terminals are extensively depleted by stimulation, synthesis only partially restores the norepinephrine concentrations. This suggests that if the rate of norepinephrine synthesis is

regulated by a feedback control mechanism, only part of the neuronal stores of norepinephrine participate in the regulation.

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