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# Electrophysiological and Pharmacological Investigation of the Superior Cervical Ganglion of the Rabbit After Preganglionic Denervation

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ELECTROPHYSIOLOGICAL AND PHARMACOLOGICAL INVESTIGATION  
OF THE SUPERIOR CERVICAL GANGLION OF THE RABBIT AFTER  
PREGANGLIONIC DENERVATION

By  
Nae J. Dun

A Dissertation Submitted to the Faculty of the Graduate School  
of Loyola University of Chicago in Partial Fulfillment  
of the Requirements for the Degree of  
Doctor of Philosophy

February

1975

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## BIOGRAPHY

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## LIST OF ABBREVIATIONS

Å	angstrom
ACh	acetylcholine
AChE	acetylcholinesterase
Ag	silver
AgCl	silver chloride
Ba	barium
°C	degrees centigrade
Ca	calcium
Carb	carbachol
ChE	cholinesterase
Cl	chloride
cm	centimeter
CO <sub>2</sub>	carbon dioxide
conc	concentration
d-Tc	d-tubocurarine
e.p.p.	endplate potential
e.p.s.p.	excitatory postsynaptic potential
E <sub>K</sub>	potassium equilibrium potential
E <sub>Na</sub>	sodium equilibrium potential
E <sub>epsp</sub>	equilibrium potential of e.p.s.p.
K	potassium
K <sub>a</sub>	dissociation constant for drug-receptor complex
Kg	kilogram
M	molar

MeCh	methacholine
m.e.p.p.	miniature endplate potential
m.e.p.s.p.	miniature excitatory postsynaptic potential
mg	milligram
Mg	magnesium
min	minute
$\mu\text{m}$	micrometer
ml	millimeter
M $\Omega$	megohm
msec	millisecond
mV	millivolt
N	number of samples in a population
Na	sodium
O <sub>2</sub>	oxygen
S.D.	standard deviation
S.E.M.	standard error of mean
sec	second
Sr	strontium
V	volt

**CHAPTER I**

**INTRODUCTION**

## CHAPTER I. INTRODUCTION

### A. ANATOMY

#### Cervical Sympathetic Trunk and Superior Cervical Ganglion

The cervical sympathetic trunk of mammals lies anterior to the transverse processes of the cervical vertebrae and behind the carotid vessels. In human, the cervical sympathetic trunk consists of three conspicuous ganglia: superior, middle and inferior cervical sympathetic ganglia. In cats and rabbits, middle cervical ganglia are frequently not present; if present, they are rather small and inconspicuous. The inferior cervical ganglion, in most instances, is partially or completely fused with the first thoracic ganglion to form the stellate ganglion (Pick, 1970).

The superior cervical ganglion, the largest of all sympathetic ganglia, is a spindle- or sometimes bell-shaped body located near the first vertebra. The preganglionic fibers of the superior cervical ganglion originate from cells located in the intermediolateral cell column of the spinal cord and enter the trunk through the upper thoracic white rami, which have their origin from T<sub>1</sub> to T<sub>4</sub> spinal segment (Ranson & Billingsley, 1918; Pick, 1970). However, there is evidence of the existence of additional cells in the spinal column that may give rise to sympathetic fibers; for example, cells along the intermediomedial cell column of the spinal cord (Pick, 1970).

The largest postganglionic trunk of superior cervical ganglion is the internal carotid nerve which follows the ramifications of the carotid artery and gives rise to the sympathetic innervation of the eyeball, lacrimal gland, mucus membrane of the nose, mouth and pharynx and many of the blood vessels of the head. The superior cardiac nerve emerges from the lower end of the ganglion and innervates the heart. Several other postganglionic trunks join the upper four spinal nerves and the cranial nerves IXth, Xth and XIIth to be distributed to the blood vessels and glands in the area supplied by these nerves (Ranson & Billingsley, 1918; Kuntz, 1945).

The superior cervical ganglion is surrounded by a capsule which consists of connective tissue cells arranged in several layers. Blood vessels and bundles of nerve fibers penetrate the surrounding capsule and branch extensively within the ganglion (Pick, 1970).

#### Sympathetic Ganglion Cells

Electron-microscopic survey of sympathetic ganglia greatly advances our present knowledge and understanding of the fine structure of sympathetic neurons (Taxi, 1957; Wyburn, 1958; Elfvin, 1963 a & b; Yamamoto, 1963; Taxi, 1969). Sympathetic ganglion cell bodies and their cell processes are covered by a sheath of satellite cells. The satellite cells encircling the neuronal elements are separated from extracellular space which is predominantly filled

with collagenous fibers by a basement membrane (Elfvin, 1963 a; Yamamoto, 1963). Although a limited number of unipolar and bipolar sympathetic neurons have been observed (Huber, 1899; Pick, 1970), the majority of neurons are multipolar with one axon and a variable number of dendrites (Ranson & Billingsley, 1918; Elfvin, 1963 a). The cell bodies of sympathetic neurons vary widely in form and size; some are oval, some are pyriform or polygonal, and their diameters range from 15 to 55  $\mu\text{m}$  (de Castro, 1932; Taxi, 1957). Several attempts have been made to classify sympathetic neurons into certain categories, using as a criterion the arrangement of Nissl granules (Hollinshead & Clark, 1935), the morphological appearance of dendrites (Dogiel, 1899), or the diameter of cells (de Castro, 1932). De Castro (1932) divided sympathetic neurons into three main types according to their cell diameters: large (35 to 55  $\mu\text{m}$ ), medium (25 to 32  $\mu\text{m}$ ) and small (15 to 22  $\mu\text{m}$ ). However, so far no evidence has been presented to relate the morphological classification of cell types to specific function of cells.

The postganglionic axon of sympathetic neurons commonly arises from the axon hillock, a cone-like area which is free from Nissl bodies (Kuntz, 1945). Axons vary considerably in diameter; they are usually nonmyelinated; myelinated axons have been occasionally observed (Ranson & Billingsley, 1918; Pick, 1970). Frequently postganglionic axons are difficult to discern from dendrites on the basis of the presence or

absence of Nissl granules (Pick, 1970). Sympathetic neurons vary within wide limits in the number and the morphological characteristics of their dendrites. The diameters of majority of dendrites are about 1 to 3  $\mu\text{m}$  when they leave the perikaryon and become much smaller in more distal portions (Elfvin, 1963 a).

#### Connection between Preganglionic and Postganglionic Neurons

Ranson and Billingsley (1918) reported that the majority of preganglionic nerve fibers that enter cat superior cervical ganglia are myelinated and their diameters vary from 1.5 to 3.5  $\mu\text{m}$ . Elfvin (1963 b) confirmed the earlier observation and reported that the preganglionic nerve fibers entering the ganglion are myelinated fibers and lose their myelin sheaths shortly before reaching the terminal arborization. The preganglionic nerve fibers run parallel to the surface of the cell body, but most frequently run parallel to or wind around a cell process and make synaptic contact with postganglionic elements in the form of axodendritic synapses, and a few axosomatic synapses. Axoaxonal synapses are encountered infrequently in sympathetic ganglia; these are synapses either between the preganglionic fibers and the axon of a ganglion cell, or between preganglionic fibers (Barton & Causey, 1958; Wyburn, 1958; Elfvin, 1963 b). In the area of synapses, the preganglionic fibers become dilated and form bouton-like structures 0.3 to 2.7  $\mu\text{m}$  in diameter (Elfvin, 1963 b).



One distinctive feature of presynaptic nerve terminals is the occurrence of closely packed vesicles. These vesicles appear in isolated form or in clusters dispersed in the nerve terminal cytoplasm. Many of these vesicles are in close proximity to the presynaptic nerve terminal membrane. Synaptic vesicles appear to be mostly agranular and measure 300 to 500 Å in diameter (Elfvin, 1963 b). These vesicles are most likely to be acetylcholine (ACh) containing vesicles and their presence constitutes the morphological evidence supporting the quantal theory of cholinergic transmission (De Robertis & Bennett, 1955). A few large dense-cored vesicles 700 to 1000 Å in diameter are regularly found in the presynaptic nerve terminals and suspected to be catecholamine containing vesicles (Grillo, 1966; Pick, 1970). In addition to the vesicular structures, numerous mitochondria, neurofilaments and opaque granules about 0.2 to 0.4 µm are found in the presynaptic nerve terminals.

In the synaptic areas, the plasma membrane of the pre- and postganglionic elements are separated by a space about 60 to 80 Å wide (Elfvin, 1963 b). Directly opposed to the presynaptic terminal boutons, a 'thickening' of the postsynaptic membrane is regularly observed in electron-micrographs (Wyburn, 1958; Elfvin, 1963 b; Pick, 1970). Little is known as to the fine structure of this 'thickening' of the postsynaptic membrane.

Accurate data on the ratio of preganglionic fibers to the ganglion cells are not available. Several studies bear-

ing on this ratio have been reported. Billingsley and Ranson (1918) counted the number of sympathetic ganglion cells of cat superior cervical ganglia and the number of myelinated fibers and reported that the ratio of preganglionic fibers to ganglion cells is approximately 1:32. Wolf (1941) included the nonmyelinated preganglionic fibers and reported the ratio to be 1:11 in one cat superior cervical ganglion and 1:17 in another preparation. However, in ciliary ganglia, the ratio of preganglionic fibers to ganglion cells was reported to be approximately 1:2 (Wolf, 1941)

#### Intra-Ganglion Chromaffin Cells

Small granular cells exhibiting an intensely bright yellow fluorescence after exposure to formaldehyde vapor were first demonstrated in rat superior cervical ganglia by Eränkö and Härkönen (1963, 1965). These cells which measure 6 to 12  $\mu\text{m}$  (Matthews & Raisman, 1969) are clearly smaller than the sympathetic neurons (15 to 55  $\mu\text{m}$ ); these small cells show remarkable similarity to the chromaffin cells of the adrenal medulla (Eränkö & Härkönen, 1965; Björklund et al., 1970). Therefore, these cells are often referred to as Small Intensely Fluorescent cells or chromaffin cells. The occurrence of chromaffin cells has been confirmed in various mammalian sympathetic ganglia (Siegrist et al., 1968; Matthews & Raisman, 1969; Williams & Palay, 1969; Björklund et al., 1970).

The chemical nature of the amine which the chromaffin cells contain has been investigated extensively (Norberg & Hamberger, 1964; Norberg et al., 1966; Bjorklund et al., 1970; Libet & Owman, 1974). Norberg et al. (1966) examined the fluorophore by means of microspectrofluorometrical techniques and suggested that in rat superior cervical ganglia the fluorophore is a primary catecholamine. More recently, it has been shown that dopamine is the principal, if not exclusive, catecholamine at least in certain mammalian sympathetic ganglia (Bjorklund et al., 1970; Libet & Owman, 1974).

Several studies on the ultrastructure of the chromaffin cells have revealed that the sympathetic preganglionic fibers make synaptic contacts with the chromaffin cells; the cell processes of chromaffin cells in turn make synaptic contacts with cell processes of sympathetic neurons (Siegrist et al., 1968; Matthews & Raisman, 1969; Williams & Palay, 1969; Matthews & Nash, 1970). The electrophysiological studies of Eccles & Libet (1961) suggested that the inhibitory potential (P wave) in rabbit superior cervical ganglia may be due to the action of epinephrine liberated from the chromaffin cells. Dunant & Dolivo (1967) and Siegrist et al. (1968) have demonstrated a similar P wave in the rat superior cervical ganglia and these authors concurred with the postulation of Eccles & Libet (1961). Most recently, Libet & Owman (1974) reported that there is a direct correlation between the intensity of the fluorescence of chromaffin cells and the amplitude of the P wave in rabbit superior cervical

ganglia. The possibility is, therefore, that these chromaffin cells may be a type of sympathetic interneurons which mediate the inhibitory process.

#### Distribution of Cholinesterase (ChE)

ChEs are a group of enzymes catalyzing the hydrolysis of choline esters. The esterase which shows the highest specificity to ACh is referred to as acetylcholinesterase (AChE) or true cholinesterase. Other esterases which show higher affinity for butyrylcholine, benzoylcholine and propionylcholine are designated butyrylcholinesterase, benzoylcholinesterase and propionylcholinesterase, respectively.

For the last few decades, the involvement of ChE in synaptic transmission has been under intensive investigation. With the advance of histochemical method, synaptic ChE can be measured qualitatively and quantitatively (Koelle, 1951). Known cholinergic neurons contain high concentrations of ChE throughout their entire structure; lower concentrations of ChE are found in non-cholinergic and adrenergic neurons (Koelle, 1951). ChE is found in excitable membrane as well as non-excitable membrane, such as erythrocyte membrane (Greig & Holland, 1949). Butyrylcholinesterase, however, is found not to associate with neuronal elements but it is restricted to the glial cells (Koelle, 1955).

By means of pharmacological methods, specifically with the use of an irreversible anti-ChE agent diisopropylphosphorofluoride (DFP) as a tool, Koelle and Koelle (1959)

distinguished two types of AChE in nerve terminals according to their orientation with respect to the nerve terminal surface membrane, the external and internal AChE. The externally oriented AChE is responsible for the rapid hydrolysis of ACh liberated during the course of synaptic transmission; therefore, it is designated as 'functional AChE.' The internal AChE represents the newly synthesized AChE.

Sawyer and Hollinshead (1945) using tissue homogenate reported that almost all ganglionic AChE is localized in presynaptic nerve terminals based on their observation that little AChE remained after chronic preganglionic denervation. Koelle (1955) confirmed and amplified this finding by histochemical methods. He demonstrated that the majority of AChE is concentrated in presynaptic nerve terminals, and only a scarce amount of AChE is detected in postganglionic elements. Furthermore, a species difference in AChE distribution has been observed. A substantial concentration of AChE in the majority of postganglionic neurons of rabbit, Rhesus monkey and human is found; a small percentage of such neurons exhibits intense AChE staining (Koelle, 1955). Sjöqvist and Fredricsson (1961) have suggested that cells stained heavily with AChE are probably those cells giving rise to the cholinergic sympathetic fibers to the sweat glands and skeletal muscle.

Ample evidence now has been accumulated indicating that approximately half of the total ganglionic AChE is associated with the preganglionic and the other half with the post-

ganglionic elements (McLennan, 1954; Dhar, 1958; Klingman & Klingman, 1969).

Morphological and Functional Changes of Sympathetic Ganglia after Preganglionic Denervation

de Castro (1930) conducted an extensive study on histological changes in cat superior cervical ganglia after denervation. Seven days after preganglionic denervation, all preganglionic fibers were fragmentized and their terminals disintegrated into granules. Barton and Causey (1958) reported that the presynaptic nerve terminals disappear seven days after preganglionic denervation of rabbit superior cervical ganglia; twelve to fourteen days later, preganglionic trunks are replaced by tube-like structures filled with Schwann cell cytoplasm. Hunt and Nelson (1965) and Hamori et al. (1968) studied the structural changes of frog and cat sympathetic ganglia after cutting presynaptic nerve fibers. The most striking event following denervation occurred at the presynaptic nerve terminals: swelling of mitochondria, dispersion of synaptic vesicles and infiltration of Schwann cell cytoplasm between presynaptic elements and postsynaptic neurons two to three days after sectioning of presynaptic fibers.

The time course of transmission failure after sectioning of preganglionic fibers of sympathetic ganglia reported from different laboratories is somewhat inconsistent. It should be borne in mind that considerable differences may be due to the choice of the species, the ganglion and the length

of the degenerating preganglionic fiber. Davidovich and Luco (1956) compared the effect of a proximal section with that of a distal section of the preganglionic nerve fibers of cat superior cervical ganglia. It was found that a 5 cm increase in the length of the degenerating preganglionic nerve delayed the failure of synaptic transmission by nearly one day.

Synaptic transmission fails before the degenerating presynaptic nerve loses the ability to conduct nerve impulses (Gibson, 1940; Davidovich & Luco, 1956; Hunt & Nelson, 1965). The failure of transmission appears to coincide with a 60% reduction in ACh content of the nerve terminals (Feldberg, 1943; Perry, 1953). One day after denervation the ACh content in the cat superior cervical ganglion declined to 60 to 90% of normal, but transmission was still intact; two days after the operation transmission began to fail, and the ACh content fell below 60% of normal; no transmission was observed on the third day and the ACh content was 10 to 15% of the normal. Gibson (1940) found a good correlation between the time course of degeneration of nerve terminals and the electrical responses of denervated sympathetic ganglia. Six days after surgical sectioning of preganglionic fibers, strong electrical stimuli applied to the preganglionic fibers failed to elicit any action potentials in the ganglion cells or in postganglionic fibers. A complete disappearance of miniature excitatory postsynaptic potentials (m.e.p.s.ps) has been reported for up to 30 days

after denervation of frog sympathetic ganglia (Hunt & Nelson, 1965) while the miniature excitatory endplate potentials (m.e.p.ps) of the frog skeletal muscle have been reported to reappear after sectioning of the motor nerve (Birks et al., 1960). Electron micrographs showed that at the denervated frog neuromuscular junction, Schwann cells migrated into the synaptic cleft and made contacts with the endplate membrane (Birks et al., 1960). They suggested that Schwann cells are responsible for the recurrence of these low frequency m.e.p.ps after degeneration of the motor nerve terminals. In denervated sympathetic ganglia, Schwann cells make similar contact with the postsynaptic membrane, but evidently failed to release any transmitter (Hunt & Nelson, 1965).

Structural changes after preganglionic denervation are reported to be confined mainly to the presynaptic elements; no discernible morphological change is found in subsynaptic structures (Sotelo, 1968; Taxi, 1969; McLachlan, 1974). McLachlan (1974) reported no significant change in soma diameter, surface area, cell volume and number of dendrites of guinea pig superior cervical ganglion cells following preganglionic denervation for periods of three to six weeks. Electron microscopy showed that the preganglionic regeneration of sympathetic, parasympathetic or somatic nerve fibers results in the formation of new nerve terminals making synaptic contacts with postganglionic elements; the appearance and distribution of these newly formed synapses are not different from those in normal ganglia (Ceccarelli et al., 1971; McLachlan, 1974).



Utilizing electrophysiological techniques, ganglionic action potentials can still be elicited from chronically denervated sympathetic neurons by direct intracellular stimulation (Hunt & Nelson, 1965; McLachlan, 1974). Normal orthodromic action potentials are restored after regenerating sympathetic fibers make synaptic contacts with sympathetic neurons which have previously been denervated by sectioning the preganglionic fibers (Gibson, 1940; Hillarp, 1946; McLachlan, 1974). Functional transmission through sympathetic ganglia is also restored following regeneration of axons originating from parasympathetic (Hillarp, 1946; Ceccarelli et al., 1971) and somatic nervous systems (McLachlan, 1974).

Electrophysiological findings on denervated sympathetic neurons seem to support the histological result that preganglionic denervation has little effect on postsynaptic elements.

## CHAPTER I. INTRODUCTION

### B. PHYSIOLOGY

#### Metabolism of Sympathetic Ganglia

The metabolism of sympathetic ganglia can be adequately measured under in vitro condition. In fact, most of the data are derived from studies on isolated preparations (Larrabee et al., 1957; Dolivo, 1974).

##### 1. Metabolism of sympathetic ganglia at rest

In the resting stage, glucose is the major substrate oxidized in isolated sympathetic ganglia. Over 50% of the glucose is oxidized rather quickly through the metabolic sequences to appear as  $\text{CO}_2$ . A fraction of the glucose is oxidized to lactate and the remaining glucose is incorporated into substances which have slow turnover rates, such as phospholipids, nucleic acids and proteins (Larrabee, 1961). The rate of ammonia production is low indicating that oxidation of amino acids is very limited in the resting stage. This is consistent with the finding that glucose is the principal source of energy in sympathetic ganglia (Dolivo, 1974).

##### 2. Metabolism of sympathetic ganglia during activity

The metabolic rate of sympathetic ganglia increases promptly when ganglion cells are activated. One feature of ganglionic metabolism that differs from that of many other peripheral nerves is a large percentage increase in oxygen

uptake at low frequency of stimulation (Larrabee, 1958; Larrabee, 1970). The rate of glucose uptake is accelerated with increasing metabolic activity (Larrabee, 1961; Dolivo, 1974). Calculated rate of glucose consumption during high frequency stimulation is not sufficient to meet the energy demand. Thus substrates other than glucose are being utilized during high metabolic activity. The rate of ammonia output is not significantly elevated during repetitive stimulation, which suggests that either sympathetic ganglion cells oxidize other endogenous substrate to sustain neuronal activity or amino acids are oxidized to some other metabolic end product not in the form of ammonia (Larrabee et al., 1957; McBride & Klingman, 1972; Dolivo, 1974).

Another significant metabolic change which accompanies the onset of activity is the acceleration of turnover of phosphatidylinositol. It has been demonstrated that the increased turnover rate of phosphatidylinositol is associated with the synaptic transmitter acting on the postsynaptic membrane because curare in concentration just adequate to block transmission completely abolishes this effect (Larrabee & Leicht, 1965). A similar observation has been reported for the rat sympathetic ganglia in situ (Larrabee, 1968). It is therefore reasonable to assume that this increased turnover of phospholipid metabolism is caused by naturally-initiated impulses in sympathetic ganglia. The physiological significance of this observation has not been explored.

### 3. Effects of glucose and oxygen lack on sympathetic ganglia

If glucose is withdrawn from the external medium for less than one hour, some deleterious change can be detected in mammalian sympathetic ganglia (Nicolescu et al., 1966). Such change in electrical and metabolic activities that occurred can be restored to normal condition upon restoration of glucose to the external medium. Two hours after glucose deprivation, sympathetic neurons show accelerated  $O_2$  consumption, peak ammonia production and gradual failure of synaptic transmission (Larrabee et al., 1957; Larrabee, 1958). Eserinized rat sympathetic ganglion cells can still respond to ACh applied extrinsically but fail to respond to preganglionic stimulation (Nicolescu et al., 1966). Electron micrographs of sympathetic ganglia placed in glucose free medium for several hours reveal that morphological change is confined mainly to presynaptic elements: swelling of the presynaptic nerve endings, sharply decreased number of mitochondria and synaptic vesicles are replaced by honeycomb-like vacuoles (Nicolescu et al., 1966). These findings in conjunction with electrophysiological evidence suggest that the primary reason for transmission failure of sympathetic ganglia in glucose free medium is presynaptic in origin (Nicolescu et al., 1966; Härkönen et al., 1969). Ribaupierre (1968) reported that in rat superior cervical ganglia, glycogen is not present in the presynaptic nerve terminals but is localized in sympathetic neurons. This observation may partially explain the very high metabolic requirement of

the presynaptic nerve terminals.

The ultimate effect of  $O_2$  lack on mammalian sympathetic ganglia is the same as deprivation of glucose. However, the transmission failure during the first two or three hours of anoxia is not due to the disintegration of presynaptic elements. Synaptic transmission gradually deteriorates when  $O_2$  is withdrawn from the external medium. Fifteen minutes after deprivation of  $O_2$ , synaptic transmission ceases but full recovery is possible even after three hours of anoxia (Dolivo et al., 1967). The early lesion is found to be chiefly restricted to the satellite cells and sympathetic neurons; the presynaptic nerve terminals are little affected (Dolivo & Rouiller, 1969). The early impairment of synaptic function may be due to alteration of the intracellular pH as a result of anaerobic metabolism of glucose (Dolivo, 1974). Extensive lysis of cellular components becomes evident five hours after depletion of oxygen and synaptic function ceases completely (Dolivo & Rouiller, 1969).

It is apparent that glucose and oxygen are essential for electrical and metabolic function in sympathetic ganglia. Furthermore, they are prerequisite for maintaining structural integrity of cellular components. However, isolated amphibian sympathetic ganglia placed in frog Ringer's solution retain apparently normal transmission for many hours in the absence of glucose and oxygen.

The Concept of Neurohumoral Transmission  
in Sympathetic Ganglia

1. Identification of neurotransmitter in sympathetic ganglia

The establishment of the role of ACh as the neurotransmitter in sympathetic ganglia is largely attributed to the work of Feldberg and his associates (Feldberg & Gaddum, 1934; Feldberg, 1943). The primary evidence supporting the contention that ACh is the putative neurotransmitter in sympathetic preganglionic neurons is the recovery and identification of ACh from the cat superior cervical ganglion perfusate (Kibjakaw, 1933; Feldberg & Gaddum, 1934; Feldberg, 1943). Several other criteria for establishing a possible role of neurotransmitter have been met beyond reasonable doubt: the synthesizing enzyme, choline acetylase is present in the presynaptic elements as is the degrading enzyme AChE which hydrolyzes ACh molecule after its release; extrinsic application of ACh closely mimics the effect of nerve impulse; the action of ACh is potentiated by anti-ChE agents. However, other biologically active substances having similar effects but which are chemically distinct from ACh, for example the esters of  $\delta$ -butyrobetanine, have been identified in the perfusate of cat superior cervical ganglia (Hosein & Proulx, 1965). Therefore, the question of whether ACh is the exclusive neurotransmitter in the sympathetic ganglion remains to be answered.

## 2. Synthesis of ACh

It is generally accepted that ACh is synthesized in preganglionic neurons and their nerve terminals. This process takes place most actively in the nerve terminal region where choline combines with acetyl-Coenzyme A catalyzed by the enzyme choline-O-acetyltransferase (ChAc) to form ACh. Ligation study has demonstrated that the enzyme ChAc is synthesized in the cell body of preganglionic neurons and transported to the nerve terminals by means of axoplasmic flow (Hebb & Waites, 1956). The substrate choline is taken up from the extracellular fluid into the nerve terminals by an active transport process which can be inhibited by a series of compounds known as hemicholiniums (Birks & MacIntosh, 1961). Adequate supply of extracellular glucose, choline and Na ions are essential for optimal synthesis of ACh (MacIntosh, 1963). The ACh content of a cat superior cervical ganglion remains relatively constant at optimal stimulation frequency of 20/sec for over an hour, suggesting the rate of synthesis can keep pace with the rate of release under the optimal condition (MacIntosh, 1963).

## 3. Storage of ACh in the nerve terminals

The results compiled thus far regarding the storage of ACh in nerve terminals are far from conclusive and irrefutable. First of all, the nerve terminals which presumably contain most, if not all, of the ACh are anatomically inaccessible for direct measurement. Secondly, in most of the studies an anti-ChE agent is added to the perfusing

medium to inhibit the hydrolysis of ACh; therefore the data so obtained are by no means physiological. Thirdly, the nerve terminals are constantly in a dynamic state; whether the techniques presently employed to measure the ACh turnover can faithfully follow this dynamic change is questionable. Nevertheless, several working hypotheses emerge from various studies of ACh storage in nerve terminals.

It is generally accepted that ACh is stored in several fractions within the nerve terminals. The first fraction which is readily released upon nerve stimulation or released spontaneously in the absence of nerve impulses is called the readily releasable fraction. In the event of continual nerve stimulation, ACh is mobilized from the second fraction called the depot ACh which serves as the reservoir to replenish the readily releasable fraction being depleted by nerve impulses. These two fractions of ACh represent approximately 85 % of the total amount of ACh in the cat superior cervical ganglion (Birks & MacIntosh, 1961). The third fraction of ACh estimated to be about 15% of the total ACh content is termed the stationary ACh (MacIntosh, 1963). This amount is not released after long-lasting stimulation; therefore, it has been suggested that the stationary ACh is located in the intraganglionic part of the preganglionic fibers rather than in their terminals. These three fractions of ACh represent the extractable total ganglionic ACh and in cat superior cervical ganglion the mean total ACh content was found to be 266 ng per ganglion (Birks & MacIntosh, 1961).



It was found that in a resting or active ganglion perfused with eserinizied medium, the ACh content gradually rises through the course of perfusion. This extra amount of ACh accumulated in this way is termed the surplus ACh. The surplus ACh is most probably a pharmacological consequence, because it represents the ACh normally hydrolyzed by AChE during regular ACh turnover. Furthermore, the surplus ACh is not released with nerve stimulations and rapidly disappears within a few minutes after AChE is reactivated (Birks & MacIntosh, 1961).

Recent studies using labelled choline and labelled ACh showed that about half of the choline resulting from the hydrolysis of ACh liberated by nerve impulses is immediately taken up into the nerve terminals and used for the resynthesis of ACh; this newly synthesized ACh is preferentially released by subsequent impulses (Collier, 1969; Collier & Lang, 1969).

#### 4. Release of ACh - evoked and spontaneous

Several aspects of the ACh liberation process are at present not fully understood, such as the transport of ACh from the cytoplasm into vesicles, the ACh content of a vesicle, the mechanism that triggers the release of vesicle from the nerve terminals and the fate of the vesicle after emptying its contents.

Experimental results have revealed several essential steps in the liberation of ACh from the nerve terminals.

When a nerve impulse arrives at the unmyelinated nerve terminal, it depolarizes the nerve terminal and triggers the release processes. The intermediate steps between the depolarization of the nerve terminal and the liberation of ACh are not known. Nevertheless, it is certain that influx of extracellular Ca into the nerve terminal is a prerequisite for this depolarization-secretion coupling. Lipicky et al. (1963) observed a slightly less than one to one molar exchange occurred between the influx of radiocalcium and the release of ACh in the rabbit superior cervical ganglion. Mg ions seem to compete with Ca ions for the critical binding site, thus inhibiting the liberation of ACh (Blackman et al., 1963 c & d).

In the absence of orthodromic stimulation, intracellular recording from both amphibian and mammalian sympathetic neurons revealed the occurrence of random, small excitatory potentials in the order of about 1 mV similar to the miniature endplate potentials (m.e.p.ps) of the neuromuscular junction (Blackman et al., 1963 c & d; Christ & Nishi, 1971 a). These spontaneously occurring potentials are called miniature excitatory postsynaptic potentials (m.e.p.s.ps). The amplitude of these m.e.p.s.ps is found to fit reasonably well with those of the unit potentials predicted by the Poisson distribution of e.p.s.p. (Christ & Nishi, 1971 a; Dun & Nishi, 1974). Therefore, it is reasonable to postulate that the synaptic transmission in sympathetic ganglia is quantal in nature.

## Passive Membrane Properties of Sympathetic Neurons

### 1. The resting membrane potential

The resting membrane potential obtained experimentally from various ganglion cells is on the average -40 to -70 mV; this value is lower than the average resting membrane potential of the spinal motoneurons, which is between -60 and -90 mV. Skok (1973) considered the observed lower resting membrane potential of the sympathetic neurons is mainly due to the physical difficulty encountered in impaling small sympathetic neurons and subsequent shunting due to imperfect sealing of neuronal membrane around the tip of the impaled microelectrode.

Woodward et al. (1969) have estimated the electrolyte distribution of rabbit superior cervical ganglia. They found the Nernst equilibrium potentials for Na, K and Cl ions are +33.6 mV, -96.6 mV and -41.1 mV, respectively; the relative permeability  $P_K : P_{Na} : P_{Cl}$  was calculated to be 1.0 : 0.06 : 0.02. Sodium and chloride ions do not contribute to the electrogenesis of resting membrane potential to any significant extent. Replacing external Na by choline or sucrose does not significantly alter the resting membrane potential (Kosterlitz et al., 1968). On the other hand, the resting membrane potential of sympathetic neurons exhibits a close relationship to the external K concentration (Blackman et al., 1963 a & b). A linear and inverse relationship is obtained between changes in the resting membrane potential and the external K concentration above

10 mM. However, a reduction of the external K concentration or total replacement of K causes only a slight increase in the resting membrane potential (Blackman et al., 1963 a & b).

## 2. Other passive membrane properties

The geometry of amphibian sympathetic neurons is relatively simple. The cells are unipolar with no dendritic branching. This enables mathematical calculation of membrane constants based on simple spherical model. However, the calculation of membrane constants of mammalian sympathetic neurons is complicated by the presence of dendrites. The current applied intracellularly to a mammalian sympathetic neuron flows through not only the soma membrane but also the axon and dendritic membranes. In addition to the soma diameter, the number and the size of cell processes and their extent of branching are essential parameters which have to be obtained prior to the calculation of membrane constants. Recent advances with the intracellular injection of fluorescent dye greatly facilitate the measurement of membrane electrical constants since the geometry of the neuron can be measured quite accurately (Nishi, 1974).

More than half of the total current applied intracellularly to the soma of a mammalian sympathetic neuron is found to flow through the axon and dendritic membranes. The total neuronal resistance is about ten times higher than that of the motoneurons; however, the specific membrane resistance is only about twice that of the motoneurons (Nishi, 1974).

Excitatory and Inhibitory Postsynaptic Potentials  
of Sympathetic Ganglion Cells

Stimulation of mammalian sympathetic ganglia in the presence of curare-like drugs gives rise to a sequence of surface potentials consisting of an initial negative (N) potential, a positive (P) potential and a late negative (LN) potential (Laporte & Lorente de nó, 1950; Eccles, 1952; Eccles & Libet, 1961; Libet, 1970); in frog sympathetic ganglia, a late late negative (LLN) potential is also observed following the LN potential (Nishi & Koketsu, 1968 a). Results obtained from intracellular recording methods have demonstrated that these surface potentials are indeed postsynaptic responses; the N, LN and LLN potentials are designated the fast excitatory postsynaptic potential (fast e.p.s.p.), slow excitatory postsynaptic potential (slow e.p.s.p.) and late slow excitatory postsynaptic potential (late slow e.p.s.p.), respectively (Eccles & Libet, 1961; Libet, 1964; Tosaka et al., 1968; Koketsu, 1969; Libet, 1970; Nishi, 1974). The P wave represents a hyperpolarizing postsynaptic potential which has been subsequently shown to be the slow inhibitory postsynaptic potential (slow i.p.s.p.) generated by a catecholamine (Eccles & Libet, 1961; Nishi & Koketsu, 1968 b; Tosaka et al., 1968; Koketsu, 1969; Libet, 1970).

1. The fast e.p.s.p.

As mentioned previously, a nerve impulse arriving at the presynaptic nerve terminals triggers the release of

ACh which diffuses across the synaptic cleft and interacts with the subsynaptic membrane. The time interval between the arrival of presynaptic impulse at the nerve terminals and the initiation of postsynaptic fast e.p.s.p. is about 1.5 to 2.0 msec (Christ & Nishi, 1971 b). In mammalian sympathetic ganglia, neurons are innervated usually with more than one preganglionic fibers; the amplitude of a fast e.p.s.p. varies from cell to cell depending on the number of synapses being activated. The moment this local-decremental potential reaches a critical level, an all-or-nothing action potential is generated from the soma membrane and propagated down its axon.

The duration of a fast e.p.s.p. of rabbit superior cervical ganglion cells ranges from 20 to 50 msec; time to peak is estimated to be 4 to 10 msec (Eccles, 1955). In guinea pig and rat superior cervical ganglion cells, the average amplitude of a fast e.p.s.p. is about 16.9 and 12.2 mV, and the average time to peak is 5.8 and 4.7 msec, respectively (Perri et al., 1970). The equilibrium potential (E<sub>e</sub>psp) is determined experimentally as the level of membrane potential at which the fast e.p.s.p. is nullified. In frog sympathetic ganglia, the equilibrium potential is estimated to be about -14 mV (Nishi & Koketsu, 1960). On the other hand, the exact value of E<sub>e</sub>psp in mammalian sympathetic ganglion cells has not been estimated because the synaptic boutons are distributed along the dendrites.

In frog sympathetic ganglion cells, Eepsp shows the following changes after altering the ionic composition of the external medium (Koketsu, 1969): a decrease in the external Na or K concentration shifts the Eepsp toward the resting membrane potential level; an increase in the external K concentration shifts the Eepsp toward the  $E_{Na}$ , and in a Cl-free solution, the Eepsp remains unchanged. It is evident, then, that during the transmitter action the sub-synaptic membrane becomes selectively permeable to Na and K ions. Thus, the ionic mechanism of the fast e.p.s.p. is similar to the electrogenesis of the e.p.p. in skeletal muscle fiber (Takeuchi & Takeuchi, 1960).

## 2. Generation of an action potential

The mode of generation of an action potential in sympathetic neurons is basically similar to other nerve cells. When the fast e.p.s.p. reaches a critical level, an action potential is initiated. The threshold level is usually 10 to 20 mV lower than the resting membrane potential. This local potential induces a physicochemical change in the adjacent cell membrane; as a result the cell membrane develops a high specific permeability to Na ions; the influx of Na ions along their electrochemical gradient carries sufficient positive charges to shift the membrane potential towards the Na equilibrium potential ( $E_{Na}$ ) momentarily. This increase in Na conductance is a function of the membrane potential. The result of this self-regenerating process is the development of an 'overshoot' type of action

potential (Hodgkin & Katz, 1949). The increase of Na conductance is only a transient event. A Na inactivation process which is still poorly understood begins to take effect shortly before the membrane potential reaches the  $E_{Na}$ . At the same time, the K efflux is accelerated over the resting level. These two combined efforts tend to return the membrane potential back to its resting level. In all cases, the membrane potential falls below the resting level and tends to move towards the K equilibrium potential ( $E_K$ ). This after-hyperpolarization is one of the prominent features of sympathetic neuron action potentials induced orthodromically, antidromically or directly through an intracellular micro-electrode.

The amplitude of the after-hyperpolarization in sympathetic neurons is on the average 20 to 30% of the spike amplitude and has a time course lasting 200 to 400 msec (Haefely, 1972). In contrast, the amplitude of the after-hyperpolarization in spinal motoneurons is only about 5 to 6 % of the spike height and lasts only 20 to 50 msec (Eccles, 1957).

The equilibrium potential of the after-hyperpolarization is between -82 and -97 mV, which is close to the  $E_K$ , indicating that the after-hyperpolarization may be due to an increase of K conductance (Lees & Wallis, 1974). According to Minota and Koketsu (1973), the enhanced and prolonged after-hyperpolarization following a tetanic stimulation is caused by an increased K conductance which is brought about



by the influx of Ca ions during repetitive spikes.

In amphibian sympathetic neurons, the threshold potential for orthodromic spike is 10 to 20 mV lower than the threshold level for the soma membrane excitation (Nishi & Koketsu, 1960). This suggests that the orthodromic spike is initiated at the axon hillock and the proximal part of the axon where threshold may be lower, as has been proposed for spinal motoneurons (Coombs et al., 1957). However, in the rabbit superior cervical ganglion cells, the synaptically induced action potential seems to arise anywhere from the soma-dendritic membrane and not restricted to the axon hillock region (Eccles, 1963).

It has been reported that frog sympathetic ganglion cells are capable of producing an action potential in a Na-free medium in which Na has been substituted isotonicly by Ca, Sr or Ba (Koketsu & Nishi, 1969). These alkaline-earth cations apparently substitute Na ions as the charge carriers and move inward during the generation of an action potential. In rabbit superior cervical ganglion cells, however, it was found that action potential can only be elicited in a solution in which Na has been replaced isotonicly by Ba; Ca, Sr or Mg renders the cell membrane inexcitable (Tashiro & Nishi, 1972). This suggests that the physico-chemical properties of mammalian sympathetic cell membrane are somewhat different from amphibian cell membrane in that its permeability to cations is more selective.

### 3. The slow e.p.s.p.

Slow excitatory postsynaptic potential (slow e.p.s.p.) can be observed in a curarized ganglion after repetitive stimulation. The slow e.p.s.p. develops after an unusually long synaptic delay of 100 to 300 msec and lasts for 2 to 3 sec (Libet, 1967). This long synaptic delay is not due to either the diffusion time for the liberated ACh to reach the postsynaptic sites where the slow e.p.s.p. is generated or the existence of an interneuron. Koketsu (1969) suggested that this long synaptic delay may in fact indicate an unusual type of cellular process involved in generating the slow e.p.s.p.

The amplitude of slow e.p.s.p. varies from cell to cell (5-20 mV); this suggests that muscarinic receptors are distributed unevenly among sympathetic neurons. In rabbit superior cervical ganglion cells, moderate hyperpolarization (10-15 mV) enhances, whereas depolarization suppresses, the slow e.p.s.p.; further hyperpolarization of 20-30 mV depresses or abolishes the slow e.p.s.p. (Kobayashi & Libet, 1968).

The ionic mechanism of slow e.p.s.p. is at present not firmly established. Alteration of external K and Na concentrations did not significantly affect the amplitude of slow e.p.s.p. as would be expected if it is generated by a change in ionic conductance (Nishi et al., 1969). Kobayashi and Libet (1968) reported there is no change in membrane conductance during the generation of slow e.p.s.p. in rabbit superior cervical ganglion cells. Metabolic inhibit-

ors such as dinitrophenol, azide and anoxia preferentially depressed the slow e.p.s.p., whereas the fast e.p.s.p. and the slow i.p.s.p. were only slightly depressed. Thus they proposed that slow e.p.s.p. is probably generated by a metabolic pump which is however entirely different from electrogenic Na-K pump or Cl pump. Weight and Votava (1970) proposed alternatively that the slow e.p.s.p. is generated by an inactivation of resting membrane K conductance; they based this hypothesis on their observation in frog sympathetic ganglion cells that the slow e.p.s.p. reverses its polarity at the membrane potential close to the K equilibrium potential; a decrease in membrane conductance is also noticed during the slow e.p.s.p.

No affirmative conclusion can be drawn at present as to the electrogenesis of the slow e.p.s.p. A metabolic process is most likely involved in the genesis of this muscarinic slow potential; in fact this may explain the nature of the unusually long synaptic delay.

#### 4. The late slow e.p.s.p.

Nishi and Koketsu (1968 a) have found a long lasting depolarization after an extremely long synaptic delay (1-5 sec) upon repetitive stimulation of B and C fibers in frog sympathetic ganglia treated with nicotine and atropine. This response (the late slow e.p.s.p.) can be recorded intracellularly from both B and C types of frog sympathetic neurons. A long exposure of the ganglion to ACh ( $1 \times 10^{-3}$  M)

that completely desensitizes both the nicotinic and muscarinic postsynaptic sites does not significantly alter the late slow e.p.s.p. This indicates that the late slow e.p.s.p. is generated by a non-cholinergic transmitter.

Unlike the slow e.p.s.p., the late slow e.p.s.p. is always accompanied by a decrease in membrane resistance. The extrapolated equilibrium potential of the late slow e.p.s.p. was approximately  $-35$  mV; this level was shifted to the hyperpolarizing direction when the external Na or K concentration was reduced; total replacement of external Cl ions with glutamate ions did not significantly alter the equilibrium potential of the late slow e.p.s.p. (Nishi, 1974). These findings suggest that the late slow e.p.s.p. is caused by the increased permeability of the non-cholinergic receptor membrane to Na and K ions (Nishi, 1974).

Chen (1971) reported the finding of a non-cholinergic late component in dog superior cervical ganglia which is quite similar in physiological and pharmacological nature to the late slow e.p.s.p. of frog sympathetic ganglia.

##### 5. The slow i.p.s.p.

The slow i.p.s.p. or the P wave recorded extracellularly was first observed by Laporte and Lorente de No (1950) in the turtle superior cervical ganglion. This synaptically mediated inhibitory potential has since been observed in the amphibian and mammalian sympathetic ganglia

(Eccles & Libet, 1961; Nishi & Koketsu, 1968 b; Tosaka et al., 1968; Libet & Kobayashi, 1969). Eccles and Libet (1961) proposed that in the rabbit superior cervical ganglion the slow i.p.s.p. is mediated by epinephrine or other catecholamine released from an adrenergic interneuron which is activated by ACh liberated from presynaptic nerve terminals. More recently, Libet (1970) suggested that dopamine is the most likely transmitter that generates the slow i.p.s.p. in the rabbit superior cervical ganglion. He demonstrated that bethanechol, a muscarinic agent, elicits a large hyperpolarization followed by a small depolarization. However, in a low Ca and high Mg solution bethanechol only elicits an enhanced depolarization; no hyperpolarization is observed. The ganglion is still responsive to catecholamine applied extrinsically in a low Ca and high Mg solution. This indicates that bethanechol acts on an interneuron to release the inhibitory transmitter rather than it is capable of hyperpolarizing the postsynaptic membrane directly.

The slow i.p.s.p. occurs in most neurons of the rabbit superior cervical ganglion with a synaptic delay of about 35 msec (Libet, 1970). The amplitude of the slow i.p.s.p. elicited by a single stimulus is very small; it is greatly augmented following a short train of supramaximal stimuli and reaches 2 to 8 mV with a duration of 10 to 30 sec. The slow i.p.s.p. is depressed and eventually abolished by

depolarization, whereas it is enhanced by moderate hyperpolarization.

The electrogenesis of slow i.p.s.p. is not entirely clear at present. Koketsu and Nishi (1967) found that the P wave in frog sympathetic ganglia is depressed by the removal of external K ions, but not affected by altering the external Cl concentrations; it is also sensitive to the blockade by ouabain, metabolic inhibitors and low temperature. Kobayashi and Libet (1968) reported no change in the amplitude of the slow i.p.s.p. after removing external K ions, and that the depressant action of ouabain is not specific, for it also depresses the fast e.p.s.p. In addition, they showed that ionic conductance does not change during the generation of slow i.p.s.p. which is in marked contrast to the electrogenesis of the i.p.s.p. of spinal motoneurons; the latter is known to be generated by an increase in K and Cl conductance (Coombs et al., 1955 b). The results, collectively, suggest that the slow i.p.s.p. may be associated with an electrogenic ion pump.

## CHAPTER I. INTRODUCTION

### C. PHARMACOLOGY

The first major study on the pharmacology of the sympathetic ganglion was conducted by Langley & Dickinson (1889) who demonstrated the stimulating and blocking actions of nicotine on cat superior cervical ganglia. Dale (1914) made the distinction between the 'nicotinic' and 'muscarinic' actions of ACh, the former an excitatory and blocking action on the autonomic ganglion and the latter an excitatory action on the parasympathetic neuro-effector junction. In 1933, Kibjakaw successfully demonstrated a technique for perfusion of cat superior cervical ganglia and reported that the effluent collected from ganglia during stimulation of the cervical sympathetic trunk contained a substance which when reinjected into the perfusion fluid caused a contraction of the nictitating membrane. This study prepared the way for an important series of experiments in Feldberg's laboratory (Feldberg & Gaddum, 1934; Feldberg & Vartiainen, 1934) that led ultimately to the identification of ACh as the neurotransmitter in the sympathetic ganglion.

In addition to the predominantly nicotinic nature of sympathetic ganglionic transmission, muscarine or muscarine-like agents were found to stimulate cat superior cervical ganglia and cause the contraction of nictitating membrane

(Koppanyi, 1932; Ambache et al., 1956; Jones, 1963; Trendelenburg & Jones, 1963). The ganglionic stimulating action of muscarine or muscarine-like agent is blocked by a small concentration of atropine but unaffected by curare or hexamethonium. ACh applied extrinsically activates both nicotinic and muscarinic receptor sites (Takehige & Volle, 1962, 1963; Koketsu et al., 1968). Surface potentials recorded on cat superior cervical ganglia after an injection of ACh exhibit triphasic wave forms resembling N, P and LN waves evoked by repetitive preganglion stimulation; methacholine, a muscarinic agonist, produces only a biphasic potential resembling P and LN waves (Takehige & Vollege, 1964 a & b). It is evident that ACh applied exogenously combines with different cholinergic receptors and mimics the action of endogenous transmitters.

#### Postsynaptic Nicotinic and Muscarinic Receptors

Definite knowledge of the structure of the cholinergic receptor is virtually non-existent. From the available evidence, it is most likely that the cholinergic receptor is a protein or phospholipid-protein complex which is a constituent of cell membrane (Ehrenpries, 1967). Despite considerable efforts by many workers for the last few decades, the molecular basis for the exhibition of different pharmacological actions of ACh has not been fully delineated. The question is often raised whether the actions of nicotinic and muscarinic agents and their respective antagonists



simply reflect the differences in the drug receptor interaction or is due to the existence of different cholinergic receptors. Ochoa and De Robertis (1973) isolated from circular smooth muscle of bovine intestine a hydrophobic fraction which show specific affinity for  $^3\text{H}$ -atropine and  $^{14}\text{C}$ -ACh, but not  $^{14}\text{C}$ -curare. The possibility is that several types of cholinergic receptors do exist.

From the studies of structure-activity of ACh and its analogues, hypothetical molecular configurations of cholinergic agonists have been proposed which show preferential complementarity to nicotinic or muscarinic receptor. All cholinergic agonists, regardless of nicotinic or muscarinic activity, possess a quaternary ammonium group which is presumably complementary to an anionic site at the cholinergic receptor (Hey, 1952; Ing, 1952; Waser, 1961; Triggle, 1965). The second feature which is common to most cholinergic agonists is an oxygen atom situated three interatomic distances from the quaternary nitrogen; this oxygen atom interacts, probably through a hydrogen bond, with the nucleophilic site of the receptor surface (Hey, 1952; Waser, 1961; Triggle, 1965). The third essential feature is the substituted group on the ether oxygen which presumably reacts with the electrophilic site of the receptor surface (Waser, 1961; Triggle, 1965). The difference between the nicotinic and muscarinic activities probably lies mainly in the characteristic interaction between the substituted moiety on the ether oxygen

characteristics of the substituted group are important factors in governing this interaction (Hey, 1952; Ing, 1952; Waser, 1961; Triggle, 1965).

The nature of muscarinic receptors has been more intensively studied than the nicotinic receptors for the reason that muscarinic agents in general show remarkable adherence to the basic molecular structure of ACh (Waser, 1961; Jones, 1963). Waser (1961) postulated that the attachment of muscarine and muscarine-like agents to the receptor involves two oxygens and a quaternary nitrogen, and that the optimal distance between the quaternary nitrogen and the ether or carbonyl oxygen is about 4 Å. Jones (1963) showed that the muscarinic agents McN-A-343, HR-602, and pilocarpine can assume configurations in which the essential atoms are located at distances similar to those postulated by Waser (1961).

#### Postsynaptic Nicotinic and Muscarinic Actions

Nicotine and related nicotinic agents initially depolarize the cell membrane by combining with nicotinic receptors; in the continual presence of these agents the cell membrane gradually repolarizes to its initial level but synaptic transmission is blocked, as has been demonstrated using surface recording or intracellular recording (Eccles, 1956; Pascoe, 1956; Ginsborg & Guerrero, 1964). Paton and Perry (1953) reported that the onset of the blocking effect of nicotine, ACh or TMA on cat superior cervical ganglia

coincides with the depolarization of the cell membrane. They theorized that the ganglionic blockade occurred after the cell membrane has returned to resting level may be due to a non-depolarizing block which resembles that produced by hexamethonium or curare. Trendelenburg (1957) demonstrated that during the late, non-depolarizing phase of nicotine blockade the ganglion regains its responsiveness to potassium chloride depolarization and to muscarinic agents. This observation supports Paton and Perry's (1953) hypothesis that the block by nicotine has a tendency to pass from a depolarizing blockade to a non-depolarizing blockade. Similar change from a depolarizing blockade induced by ACh to a non-depolarizing blockade has been reported at the endplate region of skeletal muscle (del Castillo & Katz, 1957).

A close-arterial injection of methacholine to cat superior cervical ganglia was shown to have two actions: an initial hyperpolarization and a subsequent depolarization (Takeshige et al., 1963). The blockade of transmission after an injection of methacholine was found to coincide with the hyperpolarization of the ganglion (Takeshige et al., 1963). Ginsborg (1965) utilized intracellular microelectrode techniques and demonstrated that methacholine depolarizes the frog sympathetic ganglion cell membrane and that the transmission blockade occurs at the peak of membrane depolarization. The blockade of transmission with a higher concentration of methacholine is probably a result of ganglionic

depolarization induced by methacholine reacting specifically with muscarinic receptors; hyperpolarization does not seem to be the basis of this transmission blockade (Volle, 1967). Low concentration of atropine prevents all of the actions of methacholine on the sympathetic ganglion (Pappano & Volle, 1962).

### Nicotinic Ganglionic Blocking Agents

The chemical structure of various nicotinic ganglionic blocking agents varies considerably; this again serves to illustrate the less rigid molecular nature of nicotinic receptors. The nicotinic ganglionic blocking agents are usually separated into two major categories based on their modes of action. The depolarizing blocking agents of which nicotine is the prototype act first to stimulate, then to block ganglion transmission (Paton & Perry, 1953; Triggle, 1965).

The second class of nicotinic ganglionic blocking agents is the competitive or non-depolarizing type. Non-depolarizing ganglionic blocking agents can be further subdivided into two general classes: quaternary and non-quaternary blockers. Most of the quaternary blockers are bis-onium compounds; that is, they have two quaternary nitrogens, usually separated by an alkyl chain. The bis-onium compounds have been investigated extensively because of their most unusual structure-activity relationships

as well as their potential therapeutic value (Barlow & Ing, 1948; Paton & Zaimis, 1949; Trendelenburg, 1963; Volle, 1966). The length of the alkyl chain between the two nitrogens has been shown to determine not only the site of action but also the mode of action (Barlow & Ing, 1948; Paton & Zaimis, 1949; Thesleff & Unna, 1954). When bis-onium compounds of several chain lengths were tested at the neuromuscular junction for their blocking potency, it was found that decamethonium is the most potent depolarizing blocking agent; the blocking potency decreases as the chain length is increased or decreased (Paton & Zaimis, 1949; Thesleff & Unna, 1954). However, when these same compounds were tested for blocking activity at autonomic ganglia, hexamethonium was found to be the most potent ganglionic blocker (Paton & Zaimis, 1949, 1951). Moreover, the character of the block changed from a depolarizing block at the neuromuscular junction to a non-depolarizing block at the autonomic ganglion (Paton & Zaimis, 1949, 1951). Again, this serves to reinforce the concept of specificity of receptor molecules; apparently the nicotinic receptors differ to some extent in the motor endplate and the sympathetic ganglion cell.

The principal mechanism of action of non-depolarizing agents in inhibiting ganglionic transmission is to compete effectively with ACh for occupation of postsynaptic nicotinic receptors (Lees & Nishi, 1972). However, mecamylamine, a non-depolarizing ganglionic blocker, has been shown to act presynaptically to reduce the output of ACh, in addition to

its postsynaptic inhibitory mechanism (Lees & Nishi, 1972).

### Muscarinic Blocking Agents

The muscarinic receptor blocking agents seem to show a high degree of stereospecificity. Atropine and atropine-related agents all possess structural requirements which would allow these agents to bind with the muscarinic receptor through the three point attachment scheme proposed for the binding of muscarinic agonist (Waser, 1961; Jones, 1963). The most likely points involved in the attachment of atropine molecule to the muscarinic receptor are the onium-nitrogen, the tropylium hydroxyl group and the benzyl ring (Waser, 1961).

The mechanism of action of atropine is postulated to be a competition between atropine and ACh for muscarinic receptors (Clark, 1926; Ambache, 1955; Ambache et al., 1956; Volle, 1966).

### Pharmacology of Presynaptic Cholinergic Receptor

Modification of synaptic transmission in the sympathetic ganglion by drugs acting on presynaptic nerve terminals has long been suspected. Riker and Szreniawski (1959) demonstrated that close-arterial injection of ACh to cat superior cervical ganglia initiated antidromic firings in the preganglionic nerve fibers. Dempsher and Riker (1957) reported that rat superior cervical ganglia infected with pseudorabies virus show periodic discharges of impulses along pre- and postganglionic nerve fibers.

They suggested that ACh is being released spontaneously in the preganglionic nerve terminals and is involved in the initiation of these activities. Close-arterial injection of anti-ChE agents evoked a prolonged postganglionic discharge which is not present after chronic preganglionic denervation (Takeshige & Volle, 1962). These results collectively demonstrate that the nerve terminal is pharmacologically reactive.

Riker (1968) attributed the ganglionic blocking effect of ACh in high concentration to the depolarization of the unmyelinated nerve terminals. He showed that in a small number of frog sympathetic ganglion cells transmission blockade can occur without previous depolarization of the cell membrane. Furthermore, he showed that during depolarizing transmission blockade, passage of inward current to return the membrane potential to the resting level does not restore synaptic transmission. Koketsu and Nishi (1968) using the sucrose-gap method demonstrated that ACh and nicotine applied to the intraganglionic portion of the presynaptic fibers induce a slow and transient depolarization of presynaptic nerve terminals which is blocked by curare and is not affected by atropine. As a result, they suggested that preganglionic nerve terminals are endowed with nicotinic receptors. Ginsborg (1971) confirmed this finding and in addition showed that ACh or carbachol causes only a transient reduction of nerve terminal action potentials; the presynaptic spikes in the continuous presence of these cholin-

ergic agonists regain their amplitude after a brief period of attenuation. Therefore, the cholinergic sites at the nerve terminals are probably not the source of long lasting blockade of ganglionic transmission induced by cholinomimetic agents.

Koelle (1962) has suggested that during normal ganglionic transmission the ACh initially released by nerve terminal action potentials depolarizes the nerve terminal membrane and further releases a massive amount of ACh which then acts postsynaptically to initiate postsynaptic membrane potentials. Koelle labelled this phenomenon percussive release of ACh. Nishi (1970) showed that ACh applied iontophoretically close to the presynaptic nerve terminals fails to consistently evoke m.e.p.s.ps. Direct evidence using labelled  $^3\text{H}$ -ACh showed that percussive release of ACh plays an insignificant role in the process of transmitter liberation and the amount of ACh thus liberated is far less than that released by nerve impulses (Brown et al., 1970; Collier & Katz, 1970).

The functional significance of the presynaptic cholinergic receptors is at present not certain. However, it is conceivable that they serve as a self limiting step in regulating the liberation of ACh, especially in the event of a high frequency of impulses reaching the nerve terminals.

#### Actions of Catecholamines on the Sympathetic Ganglion

The effect of catecholamines on the sympathetic ganglion has been a matter of considerable controversy; it



has been uncertain whether the ganglionic depressant action of catecholamines is due to: (1) a change in the resting membrane potential of the postganglionic neurons; (2) a decrease in the amount of ACh output, or (3) a change in the sensitivity of the postsynaptic receptors to ACh.

Early observations on the depressant action of epinephrine on the sympathetic ganglion have led many investigators to the conclusion that its principal action is to hyperpolarize the postsynaptic membrane (Marrazzi, 1939; Bulbring & Burn, 1942; De Groat & Volle, 1966). De Groat and Volle (1966) reported both inhibitory and facilitatory effects of catecholamines which were shown to be associated with hyperpolarization and depolarization of postsynaptic membrane. Moreover, they demonstrated that the hyperpolarization and depolarization induced by catecholamines are mediated by separate postsynaptic alpha- and beta-adrenergic receptors. Libet (1970) concluded the main action of catecholamines, especially dopamine, in rabbit superior cervical ganglia is to hyperpolarize the postsynaptic membrane.

A prejunctional mechanism of action has been suggested by Lundberg (1952) who concluded that the very small change in the demarcation potential of cat superior cervical ganglia could not be responsible for the inhibition of transmission by epinephrine. Others have shown that epinephrine decreases the ACh output from perfused sympathetic ganglia (Paton & Thompson, 1953; Birks & MacIntosh, 1961). Haefely (1969)

also reported a similar finding. More recently, Christ and Nishi (1971 a & b) presented evidence that the primary action of epinephrine is to reduce ACh output from presynaptic nerve terminals. They found that epinephrine attenuates the amplitude of fast e.p.s.p. without significantly altering the resting membrane potential or conductance; epinephrine decreases the frequency of m.e.p.s.ps and quantal content of evoked e.p.s.ps with little effect on the amplitude of m.e.p.s.ps and quantal size. The sensitivity of postsynaptic membrane to ACh applied iontophoretically is not altered in the presence of epinephrine. Phenoxybenzamine effectively antagonizes all the depressant actions of epinephrine. These results, collectively, indicate that the blocking action of epinephrine is exerted primarily via an alpha-adrenoceptive site on the presynaptic nerve terminals and that the weak hyperpolarization generated by catecholamines (De Groat & Volle, 1966; Libet, 1970) plays only a secondary role in the blockade of ganglionic transmission.

#### Effects of Other Substances on the Sympathetic Ganglion

A facilitatory effect of histamine and 5-hydroxytryptamine has been observed by applying these amines extrinsically to sympathetic ganglia; the facilitation is not antagonized by conventional ganglionic blocking agents (Trendelenburg, 1955, 1957; Gertner & Romano, 1961; Gertner, 1962). Sympathetic ganglia contain a considerable amount of histamine (Euler, 1949, 1966), and only a trace amount of

5-hydroxytryptamine (Gaddum & Passonen, 1955). However, the presence of these amines cannot be regarded as evidence in favor of a physiological role for these amines in sympathetic ganglia. Pharmacological findings thus far accumulated indicate that these amines act non-specifically on ganglion cell membrane or at receptors which are different from those for ACh.

Close-arterial injection of angiotensin and bradykinin to cat superior cervical ganglia caused a contraction of the nictitating membrane (Lewis & Reit, 1965). The ganglionic stimulating effect of these two polypeptides was not due to an action on the presynaptic nerve terminals since it occurred after chronic preganglionic denervation of cat superior cervical ganglia (Lewis & Reit, 1965). Another polypeptide, Substance P, potentiated the responses of cat superior cervical ganglia to submaximal preganglionic stimulation and to ACh (Beleslin et al., 1960). The mechanism of action of these polypeptides and their physiological functions, if any, have not been fully explored.

## CHAPTER I. INTRODUCTION

### D. THE AIM OF THIS STUDY

Several studies have revealed that denervated cat superior cervical ganglia respond to smaller dose of ACh (Cannon & Rosenblueth, 1936; Perry & Reinert, 1954; Ambache et al., 1956; Chein, 1960; Jones, 1963) than do normal ganglia. On the contrary, Volle and Koelle (1961), Brown (1969) and Green (1969) could not find any supersensitivity occurring in sympathetic ganglia following preganglionic denervation. In fact, they showed that denervated sympathetic ganglia become subsensitive to ACh. As a result they suggested that the sympathetic ganglion may be one of the peripheral synapses that defies Cannon and Rosenblueth's (1949) 'law of denervation supersensitivity.'

This investigation was initiated in an attempt to resolve this controversial issue. Specifically, the response of nicotinic and muscarinic receptors will be investigated systematically. Since there are two physiologically and pharmacologically distinct cholinergic receptors which show entirely different modes of activation by ACh in sympathetic neurons, it is conceivable that these two types of receptors may react in quite different manner to denervation. Several studies have suggested that the nicotinic response of sympathetic neurons is suppressed after denervation (Volle &

Koelle, 1961; Brown, 1969; Green, 1969; Vickerson & Varma, 1969), whereas others have found that denervation renders sympathetic neurons more sensitive to muscarinic depolarizing agents (Ambache et al., 1956; Bokri et al., 1963; Jones, 1963; Takeshige & Volle, 1963; Vickerson & Varma, 1969). On the other hand, Green (1969) has reported that both nicotinic and muscarinic responses are diminished after denervation.

One likely reason which may underlie these divergent results obtained by different investigators is that various techniques were employed in measuring ganglionic responses. Most of the investigators (Bokri et al., 1963; Jones, 1963; Green, 1969; Vickerson & Varma, 1969) used nictitating membrane contraction as an indicator; this method is indirect and generally inadequate to analyze the chemo-sensitivity of sympathetic ganglion cells. One of the great difficulties in the use of this method arises from the simultaneous alteration of ganglionic and neuroeffector transmissions following preganglionic denervation as has been emphasized by several investigators (Bokri et al., 1963; Langer & Trendelenburg, 1966; Langer et al., 1967; Green, 1969; Vickerson & Varma, 1969). Others have ignored the existence of two distinct cholinergic receptors in mammalian sympathetic neurons and their interpretation as a result are limited and inconclusive (Volle & Koelle, 1961;

Brown, 1969). Hence, it is the intention of this study to investigate the response of nicotinic and muscarinic receptors directly by means of the sucrose-gap method and intracellular techniques.

Virtually no attempt has been made thus far to correlate electrophysiological properties of denervated single sympathetic neurons to the pharmacological phenomenon of denervation. Hunt and Nelson (1965) and McLachlan (1974) studied the electrical properties of denervated frog and guinea pig sympathetic neurons, but they did not extend their investigations to the pharmacological aspects of denervated sympathetic neurons. Thus, the factor or factors which may contribute to the observed change of pharmacological properties of sympathetic neurons after denervation remain obscure. In this study, several factors which may be responsible for the alteration of nicotinic and muscarinic responses after denervation will be investigated at the ganglionic as well as the cellular level. Attention will be focused on five major areas: the sensitivity of nicotinic and muscarinic receptors, passive membrane properties, cell membrane excitability, equilibrium potential of nicotinic subsynaptic membrane ( $E_{\text{epsp}}$ ) and cell diameter.

Direct measurement of sensitivity of cholinceptive sites will be accomplished by applying graded concentrations of ACh and other cholinergic agonists to the ganglion and

dose-response curves of nicotinic and muscarinic actions of ACh and other agonists will then be obtained. Hunt and Nelson (1965) have reported an increase in the effective resistance of denervated frog sympathetic neurons. If a similar increase of effective resistance occurs in denervated mammalian sympathetic neurons, the resultant ACh induced depolarization will be larger; conversely, the ACh induced depolarization will become smaller if the effective resistance decreases. Therefore, passive membrane properties of ganglion cells before and after denervation will be statistically analyzed. The third area of investigation will be concerned with the excitability of cell membrane after denervation. Various physical parameters of the action potential induced by direct intracellular stimulation will be measured to determine whether or not the threshold level for cell firing is altered after denervation. The fourth area which is particularly important in the study of the change in nicotinic depolarization is the measurement of  $E_{\text{epsp}}$ . A shift of  $E_{\text{epsp}}$  to a hyperpolarizing or depolarizing direction will decrease or increase the amplitude of nicotinic depolarization. Since the  $E_{\text{epsp}}$  represents the ratio of Na and K conductance change of the subsynaptic membrane, it is of special interest to know the effect of denervation on this ratio. The fifth major area under investigation is the cell diameter. The cell diameter, to a

large extent, determines the effective resistance which, as mentioned above, is an important factor in governing the magnitude of depolarization induced by ACh. Other possible factors which may play a role in the alteration of chemosensitivity after denervation, such as the drug-receptor interaction and the density of cholinergic receptors, will also be analyzed.

The purpose for investigating these several possible factors is to determine the mechanism underlying the alteration in chemosensitivity of sympathetic neurons to various cholinergic agonists after denervation.

The majority of central cholinergic synapses have been suggested to be of the muscarinic type (cf. Karczmar et al., 1970; Phillis, 1970; Karczmar et al., 1972). Normal ganglionic transmission is predominantly nicotinic in nature; muscarinic response is more prominent in denervated ganglia than in normal ganglia as will be shown in the result section. Thus, the pharmacology of denervated ganglion cells may more closely resemble the pharmacology of central neurons. It is envisaged that the information derived from the study of denervated peripheral synapses may generate new concepts and provide better understanding of the operating mechanisms of central synapses.



## CHAPTER II

### METHODS

## CHAPTER II. METHODS

### Procedures for Chronic Preganglionic Denervation of Rabbit Superior Cervical Ganglia

Young adult white rabbits of either sex weighing 1.5 to 2.0 Kg were anesthetized with pentobarbital sodium (Nembutal), 35 mg/Kg intraperitoneally. While the animal was under surgical anesthesia, hair around the neck region was shaved with a surgical clipper and a topical antiseptic solution of Zephiran (benzalkonium chloride 1:750, Wintrop) was applied to clean the shaved area. All surgical instruments were cleaned with Zephiran solution. A small incision about 2 cm long was made in the center of the neck above the trachea; muscles underneath the incision were carefully separated to minimize any excessive bleeding. The sympathetic nerve trunk can easily be located by first identifying the vagus nerve which lies lateral to the carotid artery. Approximately 5 cm proximal to the ganglion, a segment of preganglionic sympathetic nerve trunk about 1 cm was removed. Utmost care was taken not to cut or damage any blood vessels that supply the ganglion. Achromycin V (Lederle) topical antibiotic powder was applied to the incision which was then sutured with surgical silk. Rabbits were allowed to recover from anesthesia before being returned to their cages in the animal quarter. Two to three weeks after operation, rabbits were sacrificed and the denervated ganglia were then removed for experimentation.

Methods for Testing the Success of Surgical Intervention  
of Superior Cervical Ganglia

The following methods were designed to test the success of surgical sectioning of the preganglionic fibers of the superior cervical ganglion.

1. Constricted pupil and ptosis of eyelid

The cervical sympathetic fibers which innervate the dilator muscle of the iris and the smooth muscle of the eyelid arise from the internal carotid nerve of the superior cervical ganglion. A constricted pupil and ptosis of the eyelid on the operated side as compared to the unoperated contralateral side were indicative of the successful surgical intervention of the superior cervical ganglion from its central origin.

2. Examination of the cut end

Before the ganglion was removed, the cut end was examined to ensure that no innervation or sprouting from nerves adjacent to the sympathetic trunk occurred.

3. Preganglionic testing volleys

Prior to the start of any pharmacological studies on the denervated ganglion, a few supramaximal stimuli were delivered to the preganglionic fibers. Lack of postsynaptic responses recorded with sucrose-gap method or intracellular technique was taken as the positive evidence of complete preganglionic denervation.

Kosterlitz and Wallis (1966) and Kosterlitz et al. (1968) first described the application of the sucrose-gap method in recording ganglionic potentials. The advantages of this recording method over other extracellular recording methods are as follows: (1) recording can be made during continuous perfusion of the whole ganglion; (2) large ganglionic responses can be recorded due to the high resistance of the sucrose-gap; (3) alterations in the resting membrane potential of ganglionic neurons can be detected.

#### 1. Sucrose-gap chamber

In this study, a simplified sucrose-gap arrangement was adopted to record ganglionic potentials (Nishi & Koketsu, 1968 b). The apparatus consisted of three plexi-glass chambers: T, S and P. A schematic drawing of the experimental apparatus is shown in Fig. 1. Krebs solution and isotonic sucrose solution continuously flowed through chambers T and S, respectively. Chamber P was a Krebs solution pool in which the postganglionic nerve trunk was immersed. The volume of these three chambers was approximately 1 ml each. A small notch about 0.1 mm in depth was made in the two partitions dividing these three chambers. Krebs solution flowed into chamber T via an I.D. 0.045 inch polyethylene tubing which was connected to six 3-way stopcocks placed in series. Krebs or testing solutions were delivered into separate stopcocks via rubber tubing from individual 250 ml leveling bulbs maintained 50 cm above the sucrose-gap

## SUCROSE-GAP ARRANGEMENT

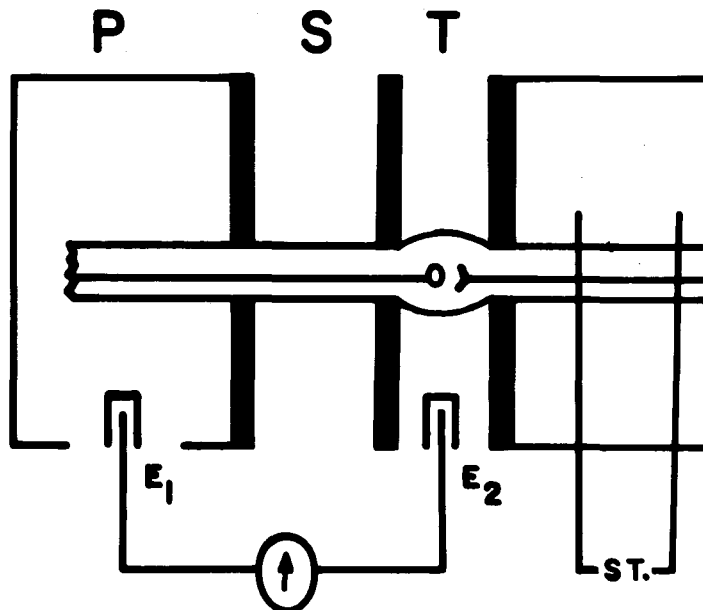


Figure 1. Diagram of the sucrose-gap arrangement. Krebs solution and isotonic sucrose solution flow through chambers T and S, respectively, chamber P is a stationary Krebs solution pool. Potential difference is measured across two agar-Ag-AgCl electrodes  $E_1$  and  $E_2$ .

chambers. The flow rate was adjusted between the last stopcock and the inflow tubing by means of a metal screw clamp, such that flow was maintained at a constant rate of about 2 ml/min. In such an arrangement, the flow was kept at a constant rate during the exchange of different solutions in any one experiment. The polyethylene tubing was coiled through a 100 ml syringe which was connected to a Haake Type FE constant temperature regulator.

Sucrose solution flowed into chamber S via a stationary 16 G needle attached to an I.D. 0.045 inch polyethylene tubing which was supplied by a 500 ml leveling bulb maintained 50 cm above the chamber. The flow rate of sucrose solution was also adjusted by means of a metal screw clamp and maintained at about the same rate as that of the Krebs solution.

## 2. Preparations

Rabbits were sacrificed by injecting 10 ml of air into the marginal ear vein. This method was preferred over using anesthetics because it is rapid (less than 30 sec from the time of injection to expiration), and devoid of any unwanted side effects. The superior cervical ganglion and its pre- and postganglionic trunks were rapidly excised and placed in a petri dish containing oxygenated Krebs solution. The connective tissue was carefully removed under a Zeiss dissecting microscope. There are usually four or five postganglionic nerve trunks which were removed leaving an internal carotid nerve intact. Thorough desheathing of the

internal carotid nerve was essential for it allowed rapid access of the isotonic sucrose solution to reach the nerve fibers and replace extracellular ions.

Before placing the preparation in the sucrose-gap chamber, a small amount of cotton was situated between the inflow tubing and the outflow of chambers T and S. This procedure ensured that a smooth continuous stream of Krebs and isotonic sucrose solution flowed through these two respective chambers. The distal end of the ganglion was positioned very close to the wall between chambers T and S. The postganglionic nerve trunk was drawn through the small notch in the wall between chambers T and S and extended across the sucrose chamber. It was further drawn through a second notch in the opposite wall between chambers S and P and there rested in chamber P which contained Krebs solution. Small amount of heavy stopcock grease (Scientific Industries) was used to seal off the two notches so that the sucrose solution was completely separated from Krebs solution. The segment of postganglionic nerve trunk in chamber S was continuously perfused with isotonic sucrose solution. Conduction of impulses along the desheathed postganglionic nerve trunk was blocked when the extracellular ions surrounding the nerve trunk were replaced by isotonic sucrose solution. This exchange process was usually completed within 20 to 30 min after the start of sucrose perfusion. The ganglion was perfused with a Krebs solution gassed with 95% O<sub>2</sub> and 5% CO<sub>2</sub>, and maintained at 30-32°C by a Haake

constant temperature regulator.

### 3. Recording and stimulating apparatus

Stable recording of ganglionic potentials could be obtained for several hours if the flow of sucrose and Krebs solution remained undisturbed. A suction electrode method was employed for orthodromic and antidromic stimulation. One of the platinum electrodes was placed inside a small glass pipette and the second platinum electrode was placed close to the first electrode but outside the glass pipette. The preganglionic or postganglionic nerve trunk was drawn into the glass pipette and immersed in the Krebs solution. Orthodromic or antidromic stimulation was applied through these two platinum electrodes. This stimulating technique was preferable to the conventional stimulating technique of immersing the nerve trunk in mineral oil since it retarded the speed of deterioration of nerve fibers. Stimulating pulses were generated by an S-8 Grass Stimulator, the output of which was isolated from ground through a Grass Isolation Unit Model SIU 5768.

Ganglionic potentials were recorded by a pair of agar-Ag-AgCl electrodes connected to a cathode follower, and then into a Nihon Kohden DC-amplifier. The potentials were displaced on a Tektronix 502A dual beam oscilloscope. The oscilloscope was precalibrated using the internal calibration source supplied by the amplifier. The potential tracings were recorded on 35 mm film using a Grass Model C-4 camera.



## Intracellular Recording and Stimulating Apparatus

### 1. Wheatstone bridge circuit

The Wheatstone bridge technique which was developed by Araki and Otani (1955) allows direct stimulation of the impaled cell through the electrode used for recording the intracellular potential change; this technique was employed later by Eccles (1955) on rabbit superior cervical ganglion cells, and by Koketsu and Nishi (1960) on frog sympathetic ganglion cells. The same technique was adopted in this study (Fig. 2).

### 2. The tissue chamber and preparations

The tissue bath was made of plexiglass with outside dimensions of 50 mm x 30 mm x 20 mm. The internal dimensions of the tissue perfusing chamber were 30 mm x 10 mm, with a fluid level of 5 mm, giving a solution volume of 1.5 cm<sup>3</sup>. The floor of the chamber was covered with Sylgard 184 Encapsulating resin (Dow Corning). This material is transparent and provided a gelatinous surface which allowed the preparation to be fixed to the floor with fine pins. The inflow was via an I.D.0.045 inch polyethylene tubing which was connected to six 3-way stopcocks as described previously.

The superior cervical ganglion with its pre- and post-ganglionic nerve trunk was removed from the rabbit and placed in a petri dish containing oxygenated Krebs solution. The preparation was carefully desheathed under a Zeiss dissecting microscope. The postganglionic nerve trunks were

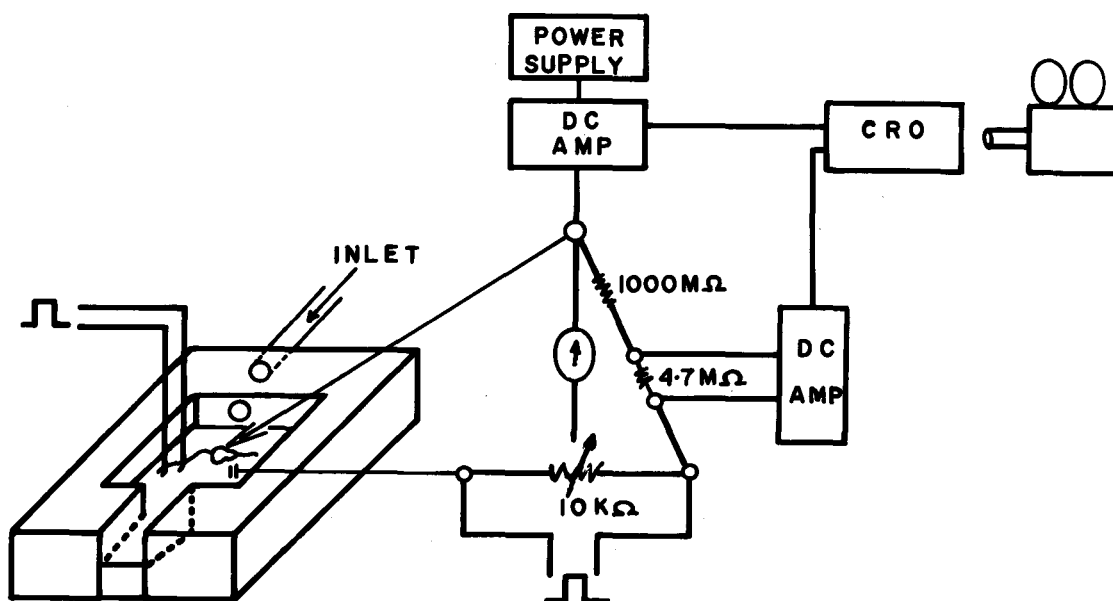


Figure 2. Diagram of the intracellular recording and stimulating apparatus. A Wheatstone bridge is incorporated in the circuit. CRO is the oscilloscope for viewing and filming. Current flowing through the circuit is measured as the potential difference across the  $4.7\text{ M}\Omega$  resistor.

removed except when antidromic stimulation was required; this procedure allowed the preparation to be fixed more securely to the floor. The preparation was then transferred to the tissue chamber and securely fixed to the floor by means of small pins about 0.05 mm in diameter. The fixing was completed with the aid of a Zeiss binocular dissecting microscope (40X). The preparation was continually superfused with a Krebs solution, gassed with 95% O<sub>2</sub> and 5% CO<sub>2</sub> and maintained at 32-34 °C by means of a Haake constant temperature regulator.

### 3. Stimulating and recording procedures

The experimental arrangement is illustrated in Fig. 2. The grid of the cathode follower was connected to the microelectrode by means of a Ag-AgCl wire inserted into the upper portion of the KCl-filled microelectrode. A calomel electrode (Beckman) was placed in the tissue bath as the indifferent electrode. Rectangular pulses were applied to the bridge circuit for direct intracellular stimulation. The suction electrode method described previously was employed for orthodromic and antidromic stimulations. Potentials were displayed on the lower beam of a Tektronix 502A dual beam oscilloscope. The current flowing through the circuit was determined by measuring the potential differences across the 4.7 M $\Omega$  resistor (Fig. 2); this potential difference was amplified by a Grass P18 DC-amplifier and displayed on the upper beam of the oscilloscope.

Intracellular recording and stimulating was accomplished with Pyrex glass capillary tubing (O.D. 1.5 mm) pulled to a fine tip using a Narishige horizontal microelectrode puller Model PN-3. The microelectrodes were then placed in a petri dish containing deionized water and inspected under a low power stage microscope. Grossly defective microelectrodes were discarded. Those microelectrodes that were suitable were filled under vacuum with absolute ethyl alcohol. Next, the alcohol was displaced first by deionized water and finally by 3 M KCl. The microelectrode resistance was tested before each experiment and only those microelectrodes having tip resistance between 30 M $\Omega$  to 50 M $\Omega$  were selected. Microelectrodes were mounted on a Narishige micro-manipulator which was positioned close to the tissue chamber.

In measuring the equilibrium potential of the fast ACh potential, two electrodes were used. One electrode consisted of a double-barreled KCl-filled microelectrode which was used for recording and passage of current (Coombs et al., 1955 a). The second electrode consisted of a single-barreled micropipette filled with 2.7M ACh having a tip resistance of 50 to 100 M $\Omega$ . This single micropipette was employed for applying ACh iontophoretically to the ganglion cells and mounted on a separate micro-manipulator. The tip of the ACh micropipette was positioned very close to the double-barreled microelectrode. A continuous negative backing current with intensity (approximately  $10^{-9}$  A) just sufficient enough to prevent the leakage of ACh was applied to the ACh

micropipette through a Ag-AgCl wire. A brief positive current pulse was applied through a Ag-AgCl wire to eject ACh from the ACh micropipette. A 300 M $\Omega$  resistor was inserted in series between the output of the isolation unit and the Ag-AgCl wire.

At the end of each experiment the coupling resistance of the double-barreled microelectrode was measured as follows. Depolarizing and hyperpolarizing current pulses were passed through the current injection barrel and the resultant potential differences across the voltage recording barrel were recorded; and a current-voltage relationship was plotted for each microelectrode. The membrane potential which was artificially altered by a given intensity of current was corrected for the potential difference due to the coupling resistance of the microelectrode.

#### Solutions and Drugs

A Krebs solution was used to superfuse the ganglion for the entire study and consisted of the following:

NaCl	117	mM
KCl	4.7	mM
CaCl <sub>2</sub>	2.5	mM
MgCl <sub>2</sub>	1.2	mM
NaHCO <sub>3</sub>	25	mM
NaH <sub>2</sub> PO <sub>4</sub>	1.2	mM
Glucose	11.5	mM

This solution was gassed with 95% O<sub>2</sub> and 5% CO<sub>2</sub> which resulted in a solution of pH 7.5.

The concentration of the sucrose solution was 315 mM (Kosterlitz et al., 1968). All the drugs used were dissolved in deionized water and diluted as required. The concentration of stock drugs was so prepared that usually 1 ml of stock drug solution when diluted to 100 ml Krebs solution will give the desired drug concentration. Acetylcholine chloride (Sigma), acetyl- $\beta$ -methylcholine chloride (Sigma), carbamylcholine chloride (Merck Sharp & Dohme) and dopamine hydrochloride (Sigma) solutions were prepared fresh on the day the experiment was performed. Other drugs were prepared as stock solution and kept in the refrigerator. The remaining drugs used were atropine sulfate (City Chemical Corporation), d-tubocurarine chloride (Sigma), hexamethonium chloride (Nutritional Biochemicals Corporation), phenoxylbenzamine hydrochloride (Smith Kline & French Laboratories), physostigmine (eserine) sulfate (Merck & Co.) and propranolol hydrochloride (Ayerst Laboratory).

### Histological Techniques

#### 1. Sectioning of the specimen - freezing method

Three weeks after sectioning the preganglionic nerve trunk, the rabbit was sacrificed; the denervated and the control ganglia were rapidly isolated and immediately frozen with Krebs solution on a freezing disc placed inside a cryostat Model CTR (International Equipment Company). The freezing method is a rapid and uncomplicated method of preparing sections and is frequently employed in preparing neurological specimens (Jones, 1966).

The frozen block of sympathetic ganglion was sectioned serially at 24  $\mu$ m thickness by a manually driven microtome. A microscope slide was placed over the microtome to transfer the frozen section to the slide. Usually four frozen sections were placed on one slide.

## 2. Fixation of the frozen section

Slides were immersed in formalin saline fixative for three to five min, and then rinsed in deionized water a few times. The formalin saline solution used was of the following composition (Jones, 1966):

36-38% Formaldehyde Solution (Merck) . . . . .	100 ml
Physiological saline (0.9%). . . . .	900 ml

## 3. Staining procedures

Ehrlich's hematoxylin and eosin staining procedure was adopted in this study (Conn et al., 1965). The staining solution was prepared by adding 3 ml of glacial acetic acid to 100 ml of Delafield Hematoxylin solution (Anderson Laboratories, Inc.). Sections were first stained in the Hematoxylin solution for 2 min and rinsed in tap water; then stained 1 min in 0.5% alcoholic Eosin solution (Anderson Laboratories, Inc.). After staining, sections were rinsed in deionized water twice and dehydrated in two changes each of 95 and 100% ethyl alcohol. Finally sections were cleaned in xylene (Baker Chemical Co.). The cleaned sections were permanently embedded with Permount mounting medium (Fisher).

#### 4. Microscopic measurement of soma diameter

The sections were viewed under a Nikon microscope (400x). The measurement of cell diameter was achieved by an eyepiece micrometer precalibrated by a stage micrometer. Only those cells with a clearly defined nucleolus were measured. Most frequently the cells appeared to be oval and spherical; therefore the average of the major and minor axes was taken as the cell diameter.

### Calculations

#### 1. Dose-response curves

Dose-response curves were obtained by plotting the mean  $\pm$  S.E.M. of responses resulting from graded concentrations of testing drugs on the ordinate versus their log concentrations on the abscissa. The values of control and denervated ganglion responses were compared using unpaired Student's  $t$ -test (Goldstein, 1971). The differences between control and denervated ganglia at a given concentration of drug were considered significant if the P value was less than 0.05.

#### 2. Calculations of membrane electrical properties

Membrane resistance was measured as follows. Hyperpolarizing current pulses of various intensities were applied through the microelectrode to displace the cell membrane potential. The current (abscissa) and resulting membrane potential change (ordinate) were plotted; the slope of the resultant line represents the total neuronal membrane



resistance. The relationship was linear when the membrane potential was displaced not beyond 50 mV from the resting membrane potential. Anomalous rectification occurs when the membrane potential is hyperpolarized beyond 50 mV from the resting level (Christ & Nishi, 1973). Only the linear portion of voltage versus current relationship was used to determine the membrane resistance.

The membrane time constant was measured as that time required for the membrane potential to rise to 63% of its maximal value when a hyperpolarizing current pulse was applied to the membrane, i.e., without the dendritic conductance correction.

The threshold potential was measured as the potential difference from the zero potential level to the threshold of an action potential. The action potential amplitude was measured as the potential difference between the resting membrane potential and the peak of the spike. The time to peak of an action potential was also measured directly from films recorded with fast sweep speed (1-2 msec/cm) as the time required from the threshold to the peak of an action potential. The total duration of a spike was measured as the time to peak plus the time of repolarization to the resting membrane potential. The amplitude of after-hyperpolarization was measured from the resting membrane potential level to the peak of after-hyperpolarization. The half duration of after-hyperpolarization was measured as the time required for the after-hyperpolarization to decay to half of its peak amplitude.

3. Measurement of equilibrium potential of the subsynaptic membrane

Equilibrium potential was determined by the method of iontophoresis of ACh. The amplitude of ACh potentials was recorded at various membrane potential levels. The membrane potential at which the ACh potential was nullified is taken as the equilibrium potential of that particular cell. The results obtained from control and denervated ganglion cells were subjected to statistical analysis.

4. Calculation of the ratio of  $\Delta g_{Na}/\Delta g_K$  of the activated subsynaptic membrane

The ratio of sodium conductance change ( $\Delta g_{Na}$ ) to potassium conductance change ( $\Delta g_K$ ) was calculated using Takeuchi and Takeuchi's (1960) equation:

$$\Delta g_{Na}/\Delta g_K = \frac{E_K - E_{\text{epsp}}}{E_{\text{epsp}} - E_{Na}}$$

where,  $E_{Na}$  and  $E_K$  are Nernst equilibrium potentials for Na and K, and values used for  $E_{Na}$  and  $E_K$  were +33.6 mV and -96.9 mV, respectively (Woodward et al., 1969).  $E_{\text{epsp}}$  is the mean equilibrium potential of the subsynaptic membrane obtained experimentally.

5. Calculation of  $K_a$ , the dissociation constant of drug-receptor complex

The dissociation constant of drug-receptor complex,  $K_a$ , was calculated from the double reciprocal plot (Goldstein et al., 1968):

$$1/\Delta = K_a/\Delta_{\text{max}} \cdot 1/x + 1/\Delta_{\text{max}}$$

where  $\Delta$ ,  $\Delta_{\text{max}}$ ,  $x$  and  $K_a$  are the membrane potential change

resulting from submaximal concentration of agonist, the maximal membrane potential change, the drug concentration and the dissociation constant of the drug-receptor complex, respectively.

The reciprocal of the change in membrane potential was plotted versus the reciprocal of drug concentrations. The y-intercept and the x-intercept of the resultant plot gives  $1/\Delta_{\max}$  and  $-1/K_a$ , respectively. The positive reciprocal of  $-1/K_a$  is the  $K_a$ .

#### 6. Drug-receptor interaction

On the assumption that the drug response is proportional to the conductance change, the drug response was calculated using the conductance change equation proposed by Takeuchi and Takeuchi (1969):

$$y = 1/(1 + K_a/A^n)$$

where

y = relative drug response

$K_a$  = dissociation constant for drug-receptor complex

A = concentration of agonist

n = number of molecules of agonist attached to a receptor. Assuming  $n = 1, 2, \dots$  etc, a theoretical curve of drug-response can be constructed by plotting y versus A. This theoretical curve was then compared with the curve obtained experimentally.

CHAPTER III

RESULTS

## CHAPTER III. RESULTS

### A. DOSE-RESPONSE CURVES OBTAINED

#### WITH SUCROSE-GAP METHOD

##### 1. Nicotinic action of ACh on control and denervated ganglia

In this series of studies, atropine ( $1 \times 10^{-5} M$ ) was added to the perfusing Krebs solution to suppress all muscarinic action of ACh. The depolarizing response so induced by the application of ACh is nicotinic in nature not contaminated by muscarinic response.

Graded concentration of ACh induced a graded nicotinic depolarization; the minimum effective concentration of ACh required to evoke a detectable nicotinic depolarization was  $1 \times 10^{-6} M$ . The rate of depolarization and the time to peak was a function of ACh concentration. At a concentration of  $1 \times 10^{-3} M$ , the ACh depolarization reached plateau in about 3 min (Fig. 3). The amplitude of the orthodromic spike gradually diminished as the membrane depolarized. At the peak of depolarization, orthodromic spikes were completely obliterated. Usually the drug was withdrawn shortly before or at the peak of depolarization. Thereafter, drug-free Krebs solution was reintroduced into the chamber. The time required for the membrane potential to return to resting level varied from preparation to preparation, usually between 10 to 20 min. Orthodromic spikes reappeared on the

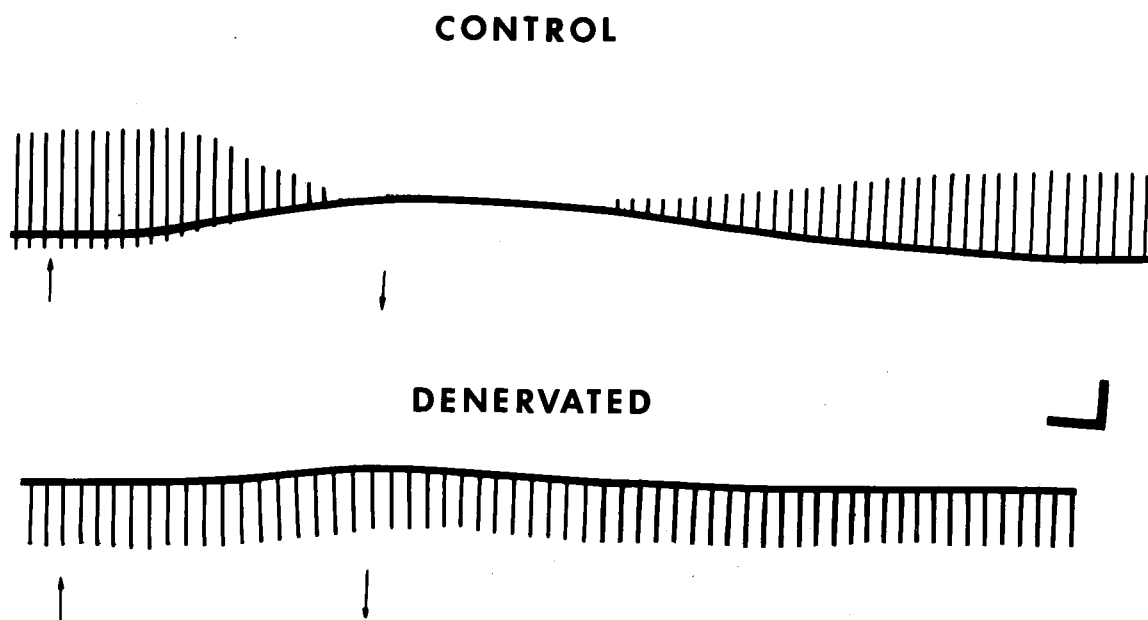


Figure 3. Sucrose-gap recordings of the effect of ACh ( $1 \times 10^{-3}$  M) on a control (upper tracing) and a denervated (lower tracing) ganglion in the presence of atropine ( $1 \times 10^{-5}$  M). Spikes in the upper tracing are orthodromic responses of the ganglion recorded by  $E_1$  electrode. Spikes (downward) in the lower tracing are antidromic responses of postganglionic trunk recorded by  $E_2$  electrode (cf. Figure 1). Arrows indicate the start and withdrawal of ACh perfusion. Calibration: 5 mV and 30 sec..

repolarizing phase. However, full recovery of orthodromic spikes usually did not occur until membrane potential had returned to resting level.

Frequently, the membrane potential fell below the resting level, as can be seen in Fig. 3. This after-hyperpolarization which followed the removal of depolarizing agent was first reported by Pascoe (1956), and later confirmed by Brown et al. (1972) and Lees and Wallis (1974) on sympathetic ganglia. The amplitude and duration of this after-hyperpolarization is found to be a function of preceding depolarization (Brown et al., 1972).

In denervated ganglia or curarized ganglia, electrical stimulation was applied to the postganglionic nerve trunk to elicit postganglionic fiber action potentials. The amplitude of the postganglionic fiber action potentials served as an index for the stability of the recording system. The amplitude of postganglionic action potentials should remain relatively constant during the course of perfusion of the ganglion. Marked fluctuation in the spike amplitude indicated that the recording system was not stable and the potential change may only be an artifact, most likely a result of uneven flow of Krebs solution and/or sucrose solution.

In atropinized denervated ganglia, the nicotinic depolarization due to ACh was found to be markedly depressed compared to control. The onset and rate of depolarization was much slower in the denervated than in the control

ganglion (Fig. 3). Applying the same concentration of ACh ( $1 \times 10^{-3} \text{M}$ ), the maximal nicotinic depolarization achieved was only half of the magnitude of the control ganglion. The time required for the membrane potential to return to initial level was shortened. No after-hyperpolarization was observed in this preparation. The after-hyperpolarization which followed the removal of the depolarizing agent was regularly observed in control ganglia. However, this drug induced after-hyperpolarization was either absent or greatly diminished in amplitude in denervated ganglia. As mentioned above, after-hyperpolarization is a function of the preceding depolarization; reduced amplitude or absence of this after-hyperpolarization indicates a diminished nicotinic depolarization.

The dose-response curves of nicotinic action of ACh on atropinized control and denervated ganglia are shown in Fig. 4. The dose-response curves show a typical sigmoidal shape. The maximal response was achieved in both preparations at the concentration of  $1 \times 10^{-3} \text{M}$ . Higher concentration of ACh caused little further increase in the drug response. The minimum effective concentration of ACh to evoke a detectable depolarization in denervated ganglia was about  $1 \times 10^{-5} \text{M}$ , which is ten times higher than the control minimum effective concentration. The maximal depolarization obtainable with each concentration of ACh was correspondingly reduced in denervated ganglia, and the difference was statistically significant. The dose-response curve shows a shift



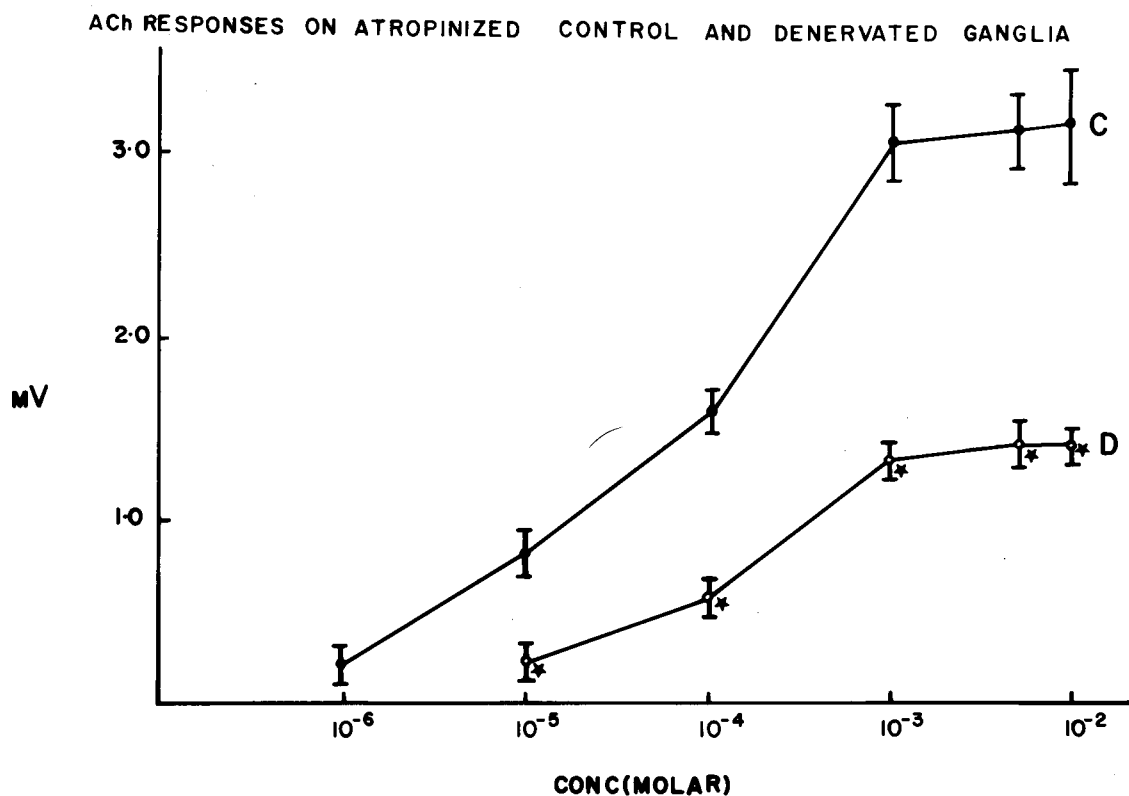


Figure 4. The dose-response curves of nicotinic responses to ACh on control and denervated ganglia in the presence of atropine ( $1 \times 10^{-5}$  M). Ordinate, depolarization in mV; abscissa, molar concentration of ACh. Each point on the curves represents the mean of results from six ganglia. Vertical bars indicate S.E.M. Asterisk denotes statistically significant difference from control ( $P < 0.05$ ) using unpaired Student's t-test.

to the right after denervation.

## 2. Muscarinic response of ACh on control and denervated ganglia

In this series of studies, and in all subsequent measurement of muscarinic responses to cholinergic agonist, d-Tc ( $5 \times 10^{-5}$  M) was added to Krebs solution to block all nicotinic responses to ACh, unless otherwise mentioned. Under such condition, the ganglionic depolarization observed after the addition of ACh is muscarinic in origin.

A low concentration of ACh ( $1 \times 10^{-6}$  M) when added to the curarized control ganglion elicited a hyperpolarizing response about 0.5 mV with negligible subsequent depolarization (Fig. 5). This initial hyperpolarization was shown to be due to the activation of muscarinic receptors on the chromaffin cells with a subsequent release of dopamine which then acts postsynaptically to generate the hyperpolarizing potential (Libet, 1970). The low amplitude postsynaptic muscarinic depolarization was masked by the initial hyperpolarizing response due to the low concentration of ACh. Phenoxybenzamine ( $1 \times 10^{-5}$  M) preferentially blocked the hyperpolarizing potential induced by a low concentration of ACh. Atropine also abolished this hyperpolarizing response.

The response of curarized denervated ganglia to a low concentration of ACh was entirely different from the control response (Fig. 5). ACh ( $1 \times 10^{-6}$  M) did not elicit any noticeable initial hyperpolarizing potential. With a rather long time delay of about 2 min after the start of ACh.

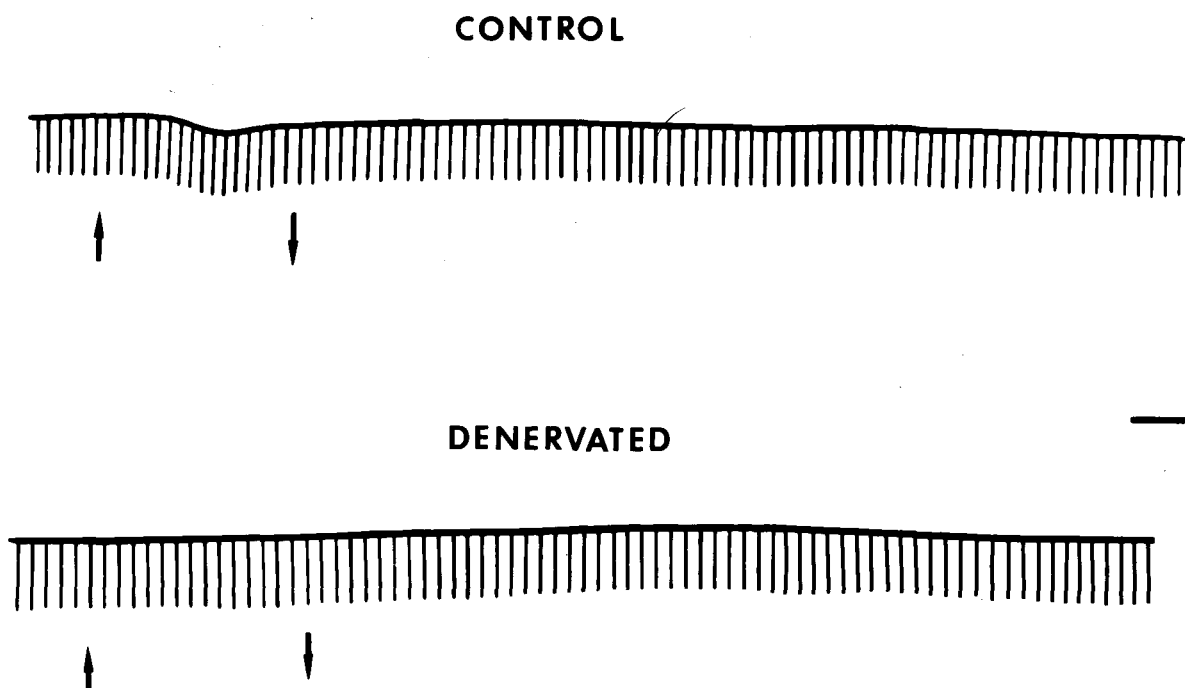


Figure 5. Sucrose-gap recordings of the effect of ACh ( $1 \times 10^{-6}$  M) in a control (upper tracing) and a denervated (lower tracing) ganglion in the presence of d-Tc ( $5 \times 10^{-5}$  M). Downward spikes represent the antidromic action potentials of the postganglionic trunk recorded by the  $E_2$  electrode. Arrows indicate the start and withdrawal of ACh perfusion.

Calibration: 2 mV and 60 sec.

( $1 \times 10^{-6} \text{M}$ ) perfusion, a slight but detectable depolarization of about 0.5 mV can be observed. In all six other denervated ganglia, ACh ( $1 \times 10^{-6} \text{M}$ ) consistently evoked a slight depolarization with no detectable initial hyperpolarizing potential.

In concentration above  $1 \times 10^{-6} \text{M}$ , ACh elicited only a depolarizing response in control ganglia and the amplitude was a function of drug concentrations. Fig. 6 depicts a typical muscarinic depolarizing response induced by  $1 \times 10^{-3} \text{M}$  ACh. The response developed much more slowly and a longer time elapsed before it reached the plateau. No after-hyperpolarization was observed following the removal of ACh. This confirms the observation of Brown et al. (1972) that muscarinic receptors are not involved in the generation of this after-hyperpolarization. The lower tracing of Fig. 6 demonstrates the effect of ACh ( $1 \times 10^{-3} \text{M}$ ) on a curarized denervated ganglion. The maximal depolarization was about twice that of the control ganglion response (upper tracing). The rate of depolarization was increased and the time needed for the membrane potential to return to resting level was prolonged. Also, no after-hyperpolarization was observed following repolarization.

The dose-response curves of curarized control and denervated ganglia to ACh are shown in Fig. 7. The maximal depolarization was about 50% greater and the minimum effective concentration was ten times lower in denervated ganglia as compared to control preparations. The dose-response

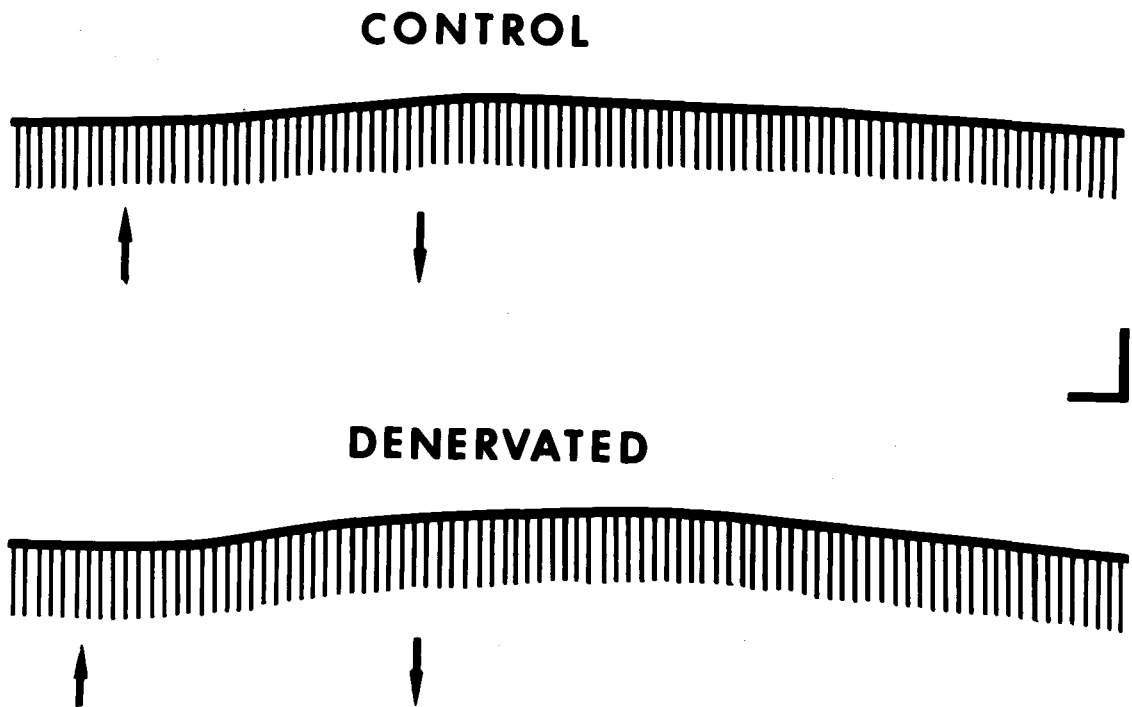


Figure 6. Sucrose-gap recordings of the effect of ACh ( $1 \times 10^{-3}$  M) on a control (upper tracing) and a denervated (lower tracing) ganglion in the presence of d-Tc ( $5 \times 10^{-5}$  M). Spikes are antidromic action potentials of the postganglionic trunk. Arrows indicate the start and withdrawal of ACh perfusion. Calibration: 5 mV and 30 sec.

## ACh RESPONSES ON CURARIZED CONTROL AND DENERVATED GANGLIA

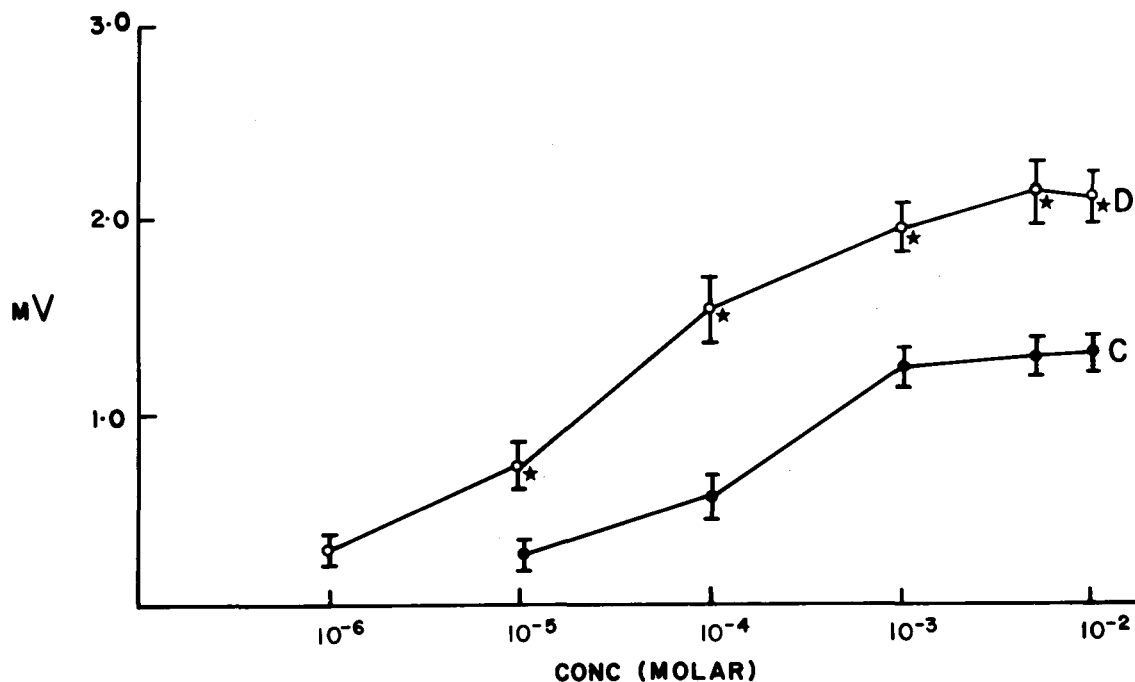


Figure 7. The dose-response curves of muscarinic responses to ACh on control and denervated ganglia in the presence of d-Tc ( $5 \times 10^{-5}$  M). Ordinate, depolarization in mV; abscissa, molar concentration of ACh. Each point on the curves represents the mean of results from six ganglia. Vertical bars indicate S.E.M. Asterisk denotes statistically significant difference from control ( $P < 0.05$ ) using unpaired Student's t-test.

relationship shifts to the left after denervation.

3. ACh responses on curarized eserized control and denervated ganglia

The ganglionic muscarinic response was shown to be augmented in the presence of an anti-ChE agent (Takeshige & Volle, 1962). In this study, eserine ( $1 \times 10^{-5} \text{M}$ ) was added to the Krebs solution in addition to d-Tc. Eserine ( $1 \times 10^{-5} \text{M}$ ) was shown to inhibit all ganglionic AChE with little direct effect on the membrane (Augustinsson, 1963).

Eserine indeed augmented the muscarinic depolarizing response to ACh in both control and denervated ganglia. In the presence of eserine, the minimum effective concentration was ten times lower, the onset and rate of depolarization was accelerated and the rate of repolarization was markedly prolonged. The increase in maximal depolarization was not as marked as other effects. Fig. 8 depicts the dose-response curves of curarized eserized control and denervated ganglia to ACh. These two curves show tendencies similar to the curarized control and denervated dose-response curves.

4. Carbachol responses on atropinized control and denervated ganglia

Carb caused a nicotinic depolarization similar to ACh. Several minor differences are noted, such as the onset and the rate of depolarization was faster, the degree of depolarization was more intense, the repolarizing phase and the after-hyperpolarization was also prolonged. Such differences can be attributed to its resistance to the enzymatic degradation by ChE (Oettingen & Eveleth, 1932; Simonart, 1932).

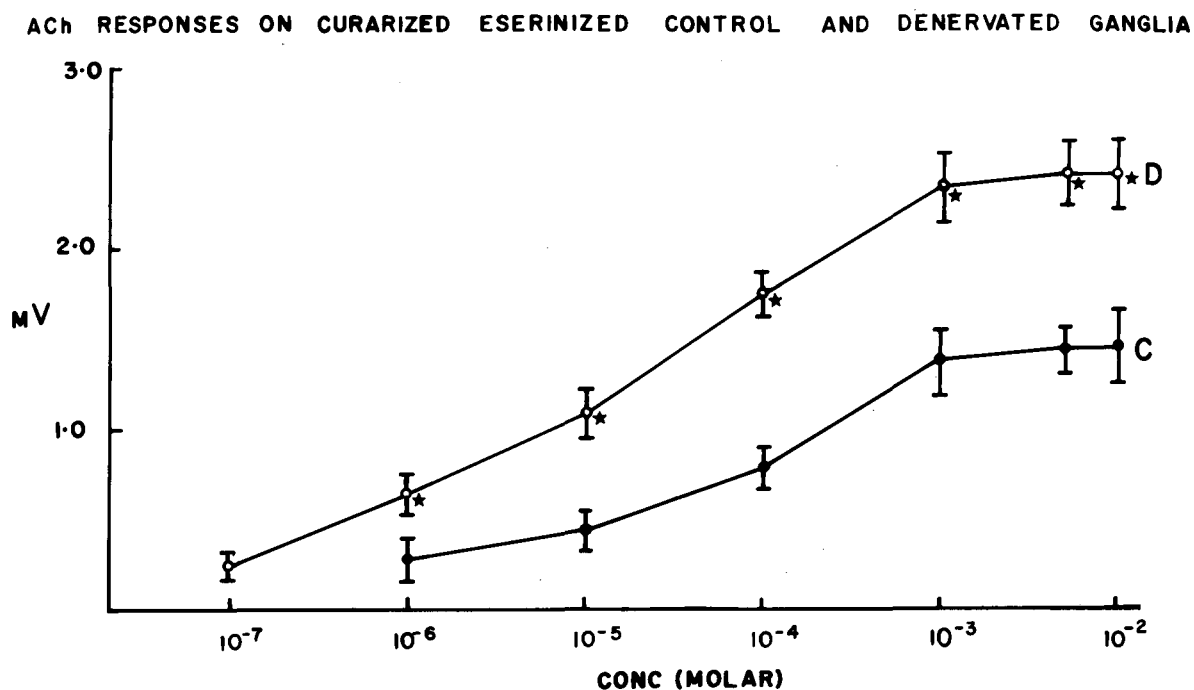


Figure 8. The dose-response curves of muscarinic responses to ACh on control and denervated ganglia in the presence of d-Tc ( $5 \times 10^{-5}$  M) and eserine ( $5 \times 10^{-5}$  M). Ordinate, depolarization in mV; abscissa, molar concentration of ACh. Each point on the curves represents the mean of results from five ganglia. Vertical bars indicate S.E.M. Asterisk denotes statistically significant difference from control ( $P < 0.05$ ) using unpaired Student's t-test.



After denervation, the nicotinic response to Carb was markedly suppressed. At the concentration of  $1 \times 10^{-3} \text{M}$ , the mean maximal depolarization attained in denervated ganglia was about 50% of the control response. The minimum effective concentration was 100 fold higher than in the control. The after-hyperpolarization which was a prominent feature in control nicotinic depolarization was either absent or greatly attenuated in denervated response. This again indicates that the nicotinic response to carbachol is substantially decreased after denervation.

Fig. 9 illustrates the dose-response curves of nicotinic action of Carb on control and denervated ganglia. The dose-response curves assumed a characteristic sigmoidal shape, and the curve shifted to the right after denervation.

#### 5. Carbachol responses on curarized control and denervated ganglia

In control ganglia, a low concentration of Carb ( $1 \times 10^{-6} \text{M}$ ) caused a slight hyperpolarizing response similar to a low concentration of ACh. However, a small depolarizing potential often followed after the hyperpolarizing potential. This probably is due to the more intense muscarinic depolarizing action of Carb which overcomes the initial hyperpolarizing response. The muscarinic action of Carb was similar to the ACh muscarinic action. Quantitative differences nevertheless were observed. The onset and rate of depolarization were faster, the duration of depolarization and repolarization was prolonged in Carb induced muscarinic

## CARB RESPONSES ON ATROPINIZED CONTROL AND DENERVATED GANGLIA

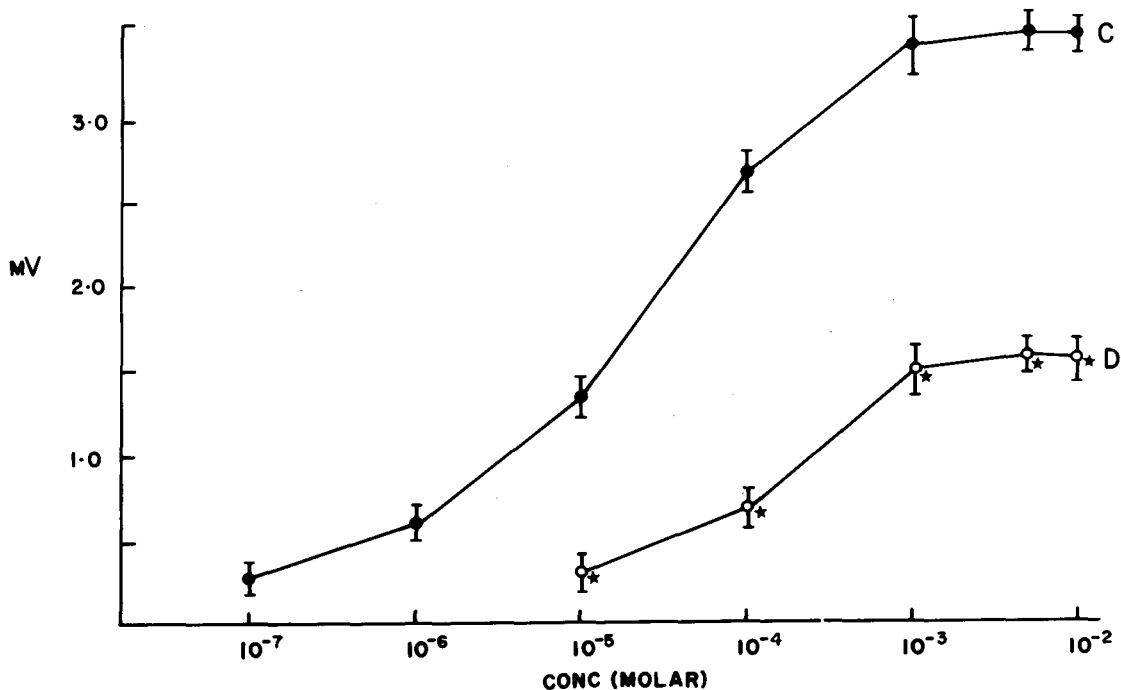


Figure 9. The dose-response curves of nicotinic responses to Carb on control and denervated ganglia in the presence of atropine ( $1 \times 10^{-5}$  M). Ordinate, depolarization in mV; abscissa, molar concentration of Carb. Each point on the curves represent the mean of results from six ganglia. Vertical bars indicate S.E.M. Asterisk denotes statistically significant difference from control ( $P < 0.05$ ) using unpaired Student's t-test.

response. The minimum effective concentration was ten fold lower and the maximal depolarization was about 10% higher than ACh response. When the Carb muscarinic response was compared to the eserinated ACh muscarinic response, the difference is insignificant.

Carb in all concentrations examined caused only a depolarizing muscarinic response on denervated ganglia. The muscarinic response was markedly enhanced after denervation as evident by nearly 60% increase in maximal depolarization and a ten fold reduction in minimum effective concentration. In each concentration level the muscarinic response before and after denervation was found to be significantly different (Fig. 10).

Again, no after-hyperpolarization was observed following the removal of Carb in either the control or the denervated ganglion.

#### 6. Methacholine responses on control and denervated ganglia

MeCh, a specific muscarinic agonist, was used to ascertain whether the enhanced response observed after denervation was truly muscarinic in nature. MeCh was found to have little or no nicotinic effect on sympathetic ganglia (Volle, 1967). Hence, d-Tc was not used to block nicotinic responses in this series of experiments. This offered an opportunity to study the muscarinic response not contaminated with nicotinic blocking agent and at the same time to compare the MeCh muscarinic response quantitatively to the

## CARB RESPONSES ON CURARIZED CONTROL AND DENERVATED GANGLIA

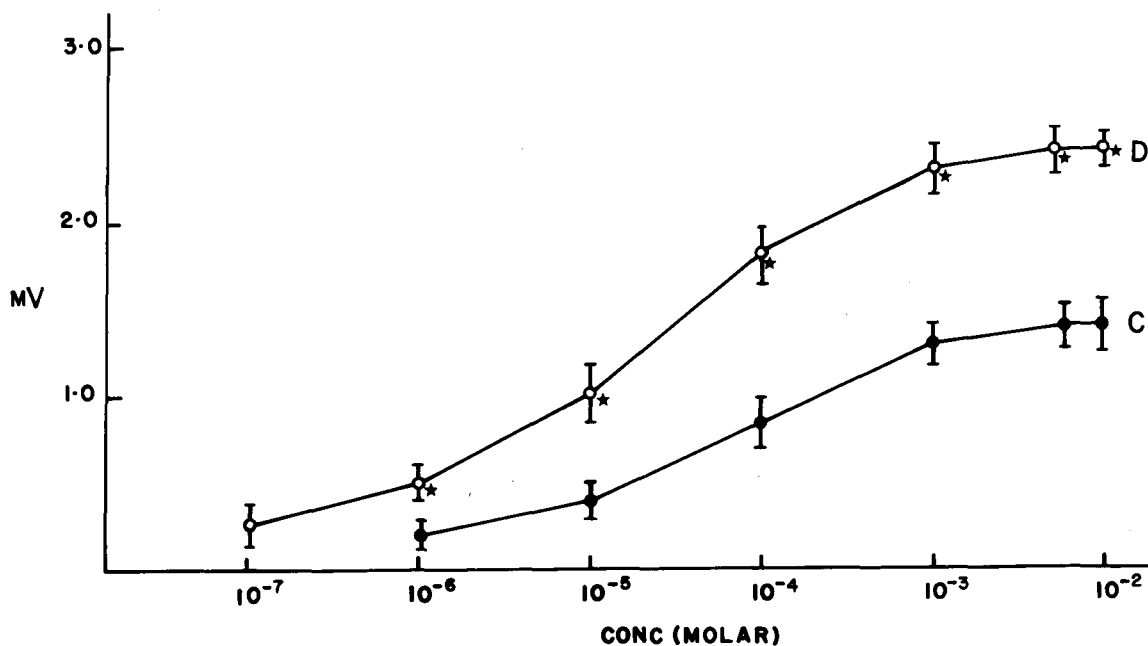


Figure 10. The dose-response curves of muscarinic responses to Carb on control and denervated ganglia in the presence of d-Tc ( $5 \times 10^{-5}$  M). Ordinate, depolarization in mV; abscissa, molar concentration of Carb. Each point on the curves represent the mean of results from six ganglia. Vertical bars indicate S.E.M. Asterisk denotes statistically significant difference from control ( $P < 0.05$ ) using unpaired Student's t-test.

ACh muscarinic response after nicotinic suppression.

In control ganglia, MeCh ( $1 \times 10^{-6} \text{M}$ ), similar to ACh ( $1 \times 10^{-6} \text{M}$ ), elicited a low amplitude hyperpolarization of about 0.5 mV with no subsequent detectable depolarizing response. Concentrations of MeCh above  $1 \times 10^{-6} \text{M}$  evoked only a depolarizing potential. A representative recording of the action of MeCh ( $1 \times 10^{-3} \text{M}$ ) on a control ganglion is shown in Fig. 11. MeCh depolarized the cell membrane; as a result, the amplitude of the orthodromic spike decreased and the positive afterpotential was attenuated. The magnitude of the membrane depolarization was not sufficient to completely abolish the orthodromic spike. The onset, amplitude and duration of MeCh induced depolarization was quantitatively similar to ACh muscarinic depolarization. Repolarization was marked by a gradual regaining of spike height and positive afterpotential.

MeCh ( $1 \times 10^{-6} \text{M}$ ) evoked in denervated ganglia only a depolarizing response not preceded by a hyperpolarizing response, similar to ACh ( $1 \times 10^{-6} \text{M}$ ). Fig. 11 illustrates the action of MeCh ( $1 \times 10^{-3} \text{M}$ ) on a denervated ganglion. The maximal depolarization increased more than 50% over the control response. The rate and duration of depolarization was also increased in the denervated ganglion. The depolarization induced by MeCh in both control and denervated ganglia was completely abolished by atropine ( $1 \times 10^{-5} \text{M}$ ).

After-hyperpolarization was observed in neither control nor denervated ganglia after MeCh was withdrawn from the

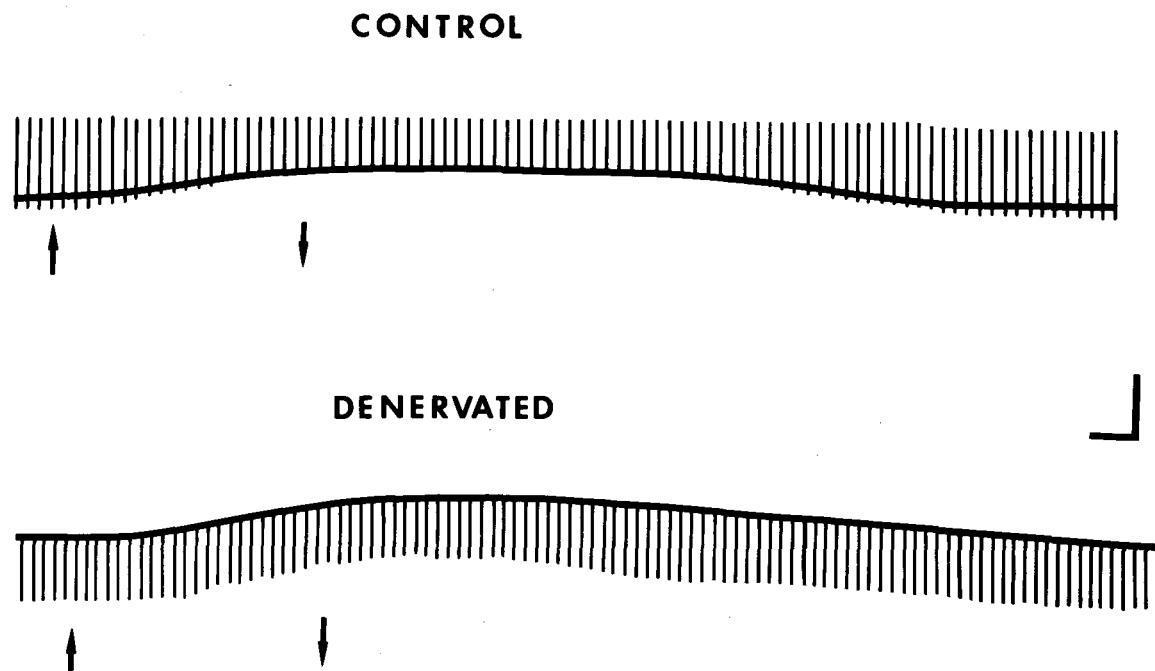


Figure 11. Sucrose-gap recordings of the effect of MeCh ( $1 \times 10^{-3}$  M) on a control (upper tracing) and a denervated (lower tracing) ganglion. Spikes in the upper and lower records are orthodromic ganglionic responses and antidromic postganglionic trunk responses, respectively. Arrows indicate the start and withdrawal of MeCh perfusion. Calibration: 5 mV and 60 sec.

perfusing solution.

Fig. 12 demonstrates the dose-response curves of MeCh on control and denervated ganglia. The mean maximal depolarization in denervated ganglia was 50% higher than the mean maximal depolarization in control ganglia. The minimum effective concentration was ten times less in denervated ganglia. The maximal depolarization was slightly higher and the slope was steeper in MeCh dose-response curves as compared to the ACh muscarinic dose-response curves. This is probably due to the fact that MeCh is hydrolyzed only by specific cholinesterase at a slower rate (Oettingen & Eveleth, 1932; Simonart, 1932). The MeCh dose-response curve exhibited a parallel shift to the left after denervation.

7. The effect of dopamine on control and denervated ganglia

Dopamine in concentration of  $1 \times 10^{-6}$  to  $1 \times 10^{-3} \text{M}$  produced a hyperpolarizing potential of various intensity. Dopamine ( $1 \times 10^{-6} \text{M}$ ) caused only a slight hyperpolarization which in two out of six control ganglia was inconspicuous, whereas all six denervated ganglia responded to dopamine ( $1 \times 10^{-6} \text{M}$ ) with a detectable hyperpolarizing potential. The dopamine dose-response curves in both control and denervated ganglia reached the plateau at a concentration of  $1 \times 10^{-4} \text{M}$ .

Fig. 13 depicts the effect of dopamine ( $1 \times 10^{-4} \text{M}$ ) on a control ganglion. Two min after the start of dopamine perfusion, the membrane potential began to increase with a concomitant decrease in orthodromic spike amplitude. The

## MeCh RESPONSES ON CONTROL AND DENERVATED GANGLIA

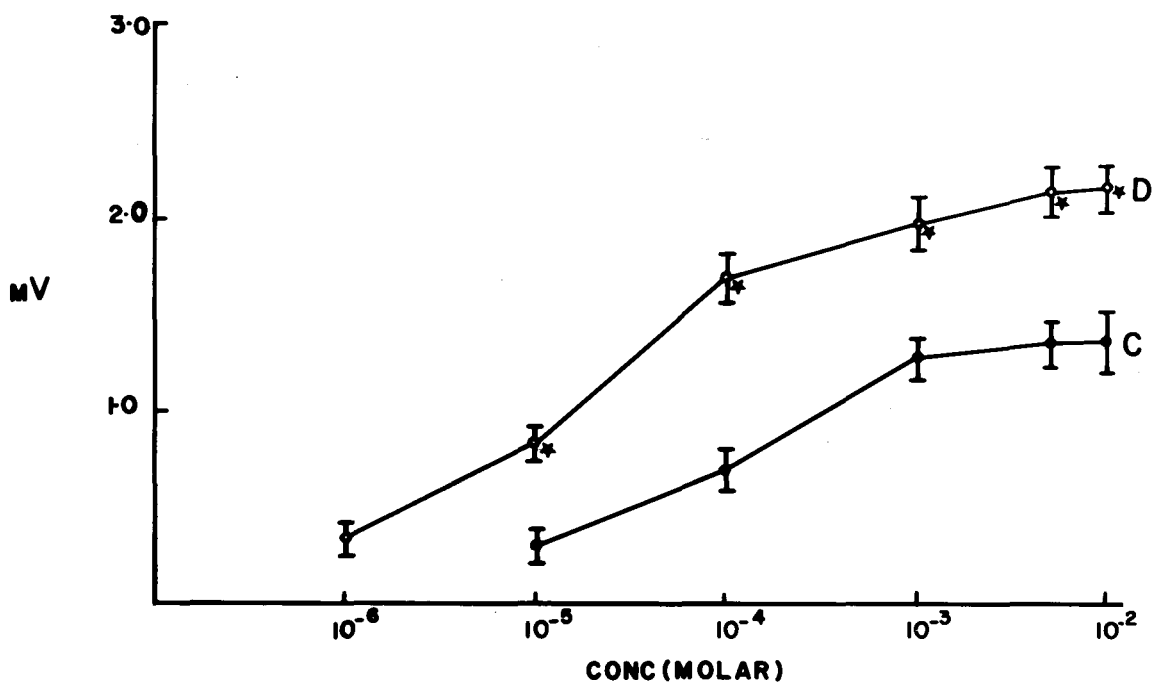


Figure 12. The dose-response curves of muscarinic responses to MeCh on control and denervated ganglia. Ordinate, depolarization in mV; abscissa, molar concentration of meCh. Each point on the curves represents the mean of results from five ganglia. Vertical bars indicate S.E.M. Asterisk denotes statistically significant difference from control ( $P < 0.05$ ) using unpaired Student's t-test.



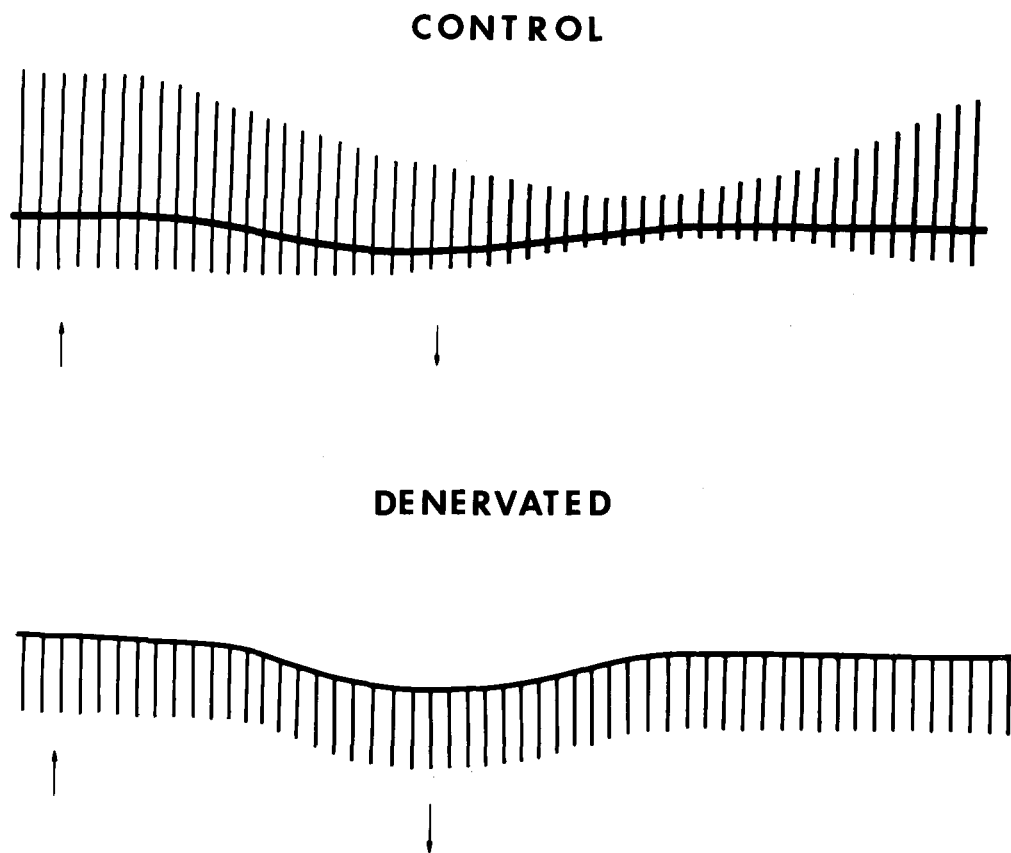


Figure 13. Sucrose-gap recordings of the effect of dopamine ( $1 \times 10^{-4}$  M) on a control (upper tracing) and a denervated (lower tracing) ganglion. Spikes in the upper and lower records are orthodromic ganglionic responses and antidromic postganglionic trunk action potentials, respectively. Arrows indicate the start and withdrawal of dopamine perfusion. Calibration: 2 mV and 60 sec.

hyperpolarizing potential reached its plateau (1.0 mV) in about 2 min; thereafter, the membrane potential spontaneously repolarized even in the continual presence of dopamine. The repolarizing phase was usually completed within 3 min. However, orthodromic action potentials continued to be depressed after the membrane potential had returned to the resting level. This clearly indicates that the depression of orthodromic spikes is not entirely due to the hyperpolarizing action of dopamine. Indeed, as will be demonstrated later in the intracellular study of dopamine, the depression of synaptic transmission is predominantly a presynaptic event.

The denervated ganglion was more sensitive to the hyperpolarizing effect of dopamine. Dopamine ( $1 \times 10^{-4} M$ ) produced a slightly larger hyperpolarization in the denervated ganglion (Fig. 13). In this preparation, the hyperpolarizing potential reached about 1.2 mV in amplitude. The membrane potential, similar to the control ganglion, spontaneously repolarized. The time course of the hyperpolarizing and the repolarizing phases paralleled closely the time course of the control dopamine response.

The dose-response curves of dopamine on control and denervated ganglia are shown in Fig. 14. There is an apparent increase in the sensitivity of denervated ganglia to dopamine as is evidenced by a shift of the dose-response curve to the left. However, statistical analysis fails to reveal any significant difference.

## DOPAMINE RESPONSES ON CONTROL AND DENERVATED GANGLIA

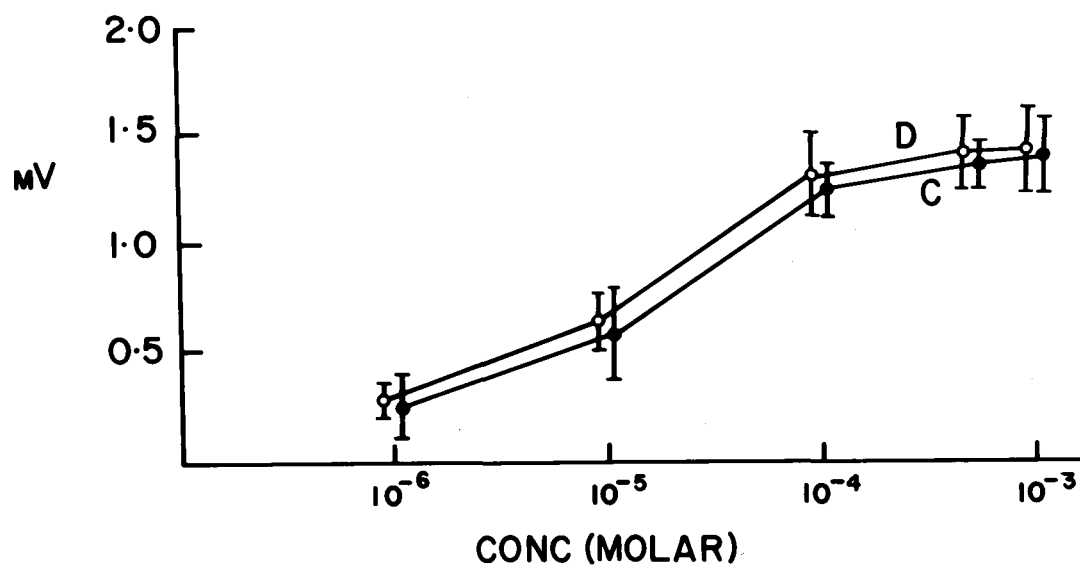


Figure 14. The dose-response curves of control and denervated ganglia to dopamine. Ordinate, hyperpolarization in mV; abscissa, molar concentration of dopamine. Each point on the curves represents the mean of results from six ganglia. Vertical bars indicate S.E.M.

## CHAPTER III. RESULTS

### B. DOSE-RESPONSE CURVES OBTAINED

#### WITH INTRACELLULAR TECHNIQUE

##### 1. ACh response on atropinized control and denervated ganglion cells

Ganglionic muscarinic response to ACh was suppressed by the addition of atropine ( $1 \times 10^{-5} \text{M}$ ).

ACh in concentration between  $1 \times 10^{-5}$  and  $1 \times 10^{-2} \text{M}$  when added to control ganglion cells caused almost an immediate depolarization of the cell membrane. The magnitude and rapidity of nicotinic depolarization varied markedly from cell to cell. Fig. 15 illustrates the response of a single ganglion cell to ACh ( $1 \times 10^{-3} \text{M}$ ). Less than 15 sec after the beginning of ACh perfusion, cell membrane showed a rapid depolarization which reached its peak in less than 30 sec. Orthodromic spikes were immediately depressed as the cell membrane depolarized and completely disappeared at the peak of membrane depolarization. The depolarizing action of ACh terminated soon after ACh was withdrawn and drug-free Krebs solution reintroduced into the tissue bath. Orthodromic spikes reappeared as the membrane repolarized; however, the spike did not regain its full amplitude after the membrane potential had returned to resting level. At a concentration of ACh ( $1 \times 10^{-3} \text{M}$ ), the depolarization ranged from 15 to 37 mV in eight cells studied. The after-hyperpolarization which

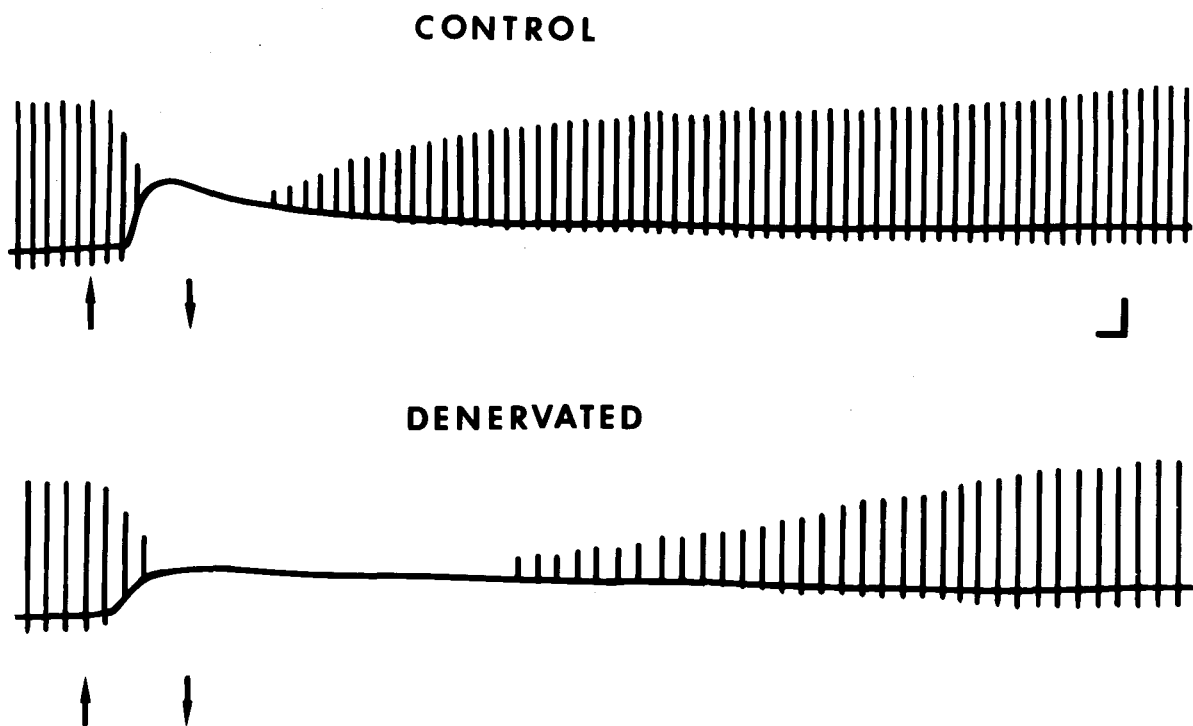


Figure 15. Intracellular recordings of the effect of ACh ( $1 \times 10^{-3}$  M) on a control (upper tracing) and a denervated (lower tracing) ganglion cell in the presence of atropine ( $1 \times 10^{-5}$  M). Spikes in the upper record are orthodromic action potentials. Spikes in the lower record are action potentials elicited by direct intracellular stimulation. Arrows indicate the start and withdrawal of ACh perfusion. Calibration: 15 mV and 10 sec.

occurred after removal of depolarizing agents in sucrose-gap preparation was not detected from recordings of single cell response. It is possible that the after-hyperpolarization generated by a single neuron is small and is difficult to detect. In sucrose-gap method, the after-hyperpolarization observed is the algebraic sum of responses of the whole population of ganglion cells.

The nicotinic depolarization was always accompanied by a marked decrease in membrane resistance as measured by the amplitude of repetitively induced electrotonic potentials produced by inwardly directed constant current pulses.

Fig. 15 shows the effect of ACh ( $1 \times 10^{-3} \text{M}$ ) on a denervated ganglion cell. The rate of depolarization was slower and the maximal amplitude was much lower compared to the control response. The rate of repolarization was also slower; this was probably due to the partial disappearance of ganglionic AChE. Action potentials induced by direct intracellular stimulation were abolished at the peak of depolarization. The nicotinic response of seven denervated ganglion cells to ACh ( $1 \times 10^{-3} \text{M}$ ) did not vary markedly as reflected by smaller S.E.M. of the dose-response relationship. Apparently the nicotinic receptor sensitivity was generally depressed after denervation.

The dose-response curves of nicotinic action of ACh on control and denervated ganglion cells are depicted in Fig. 16. The mean maximal depolarization decreased nearly 50% and the minimum effective concentration was elevated ten fold

ACH RESPONSES ON ATROPINIZED CONTROL AND DENERVATED GANGLION CELLS

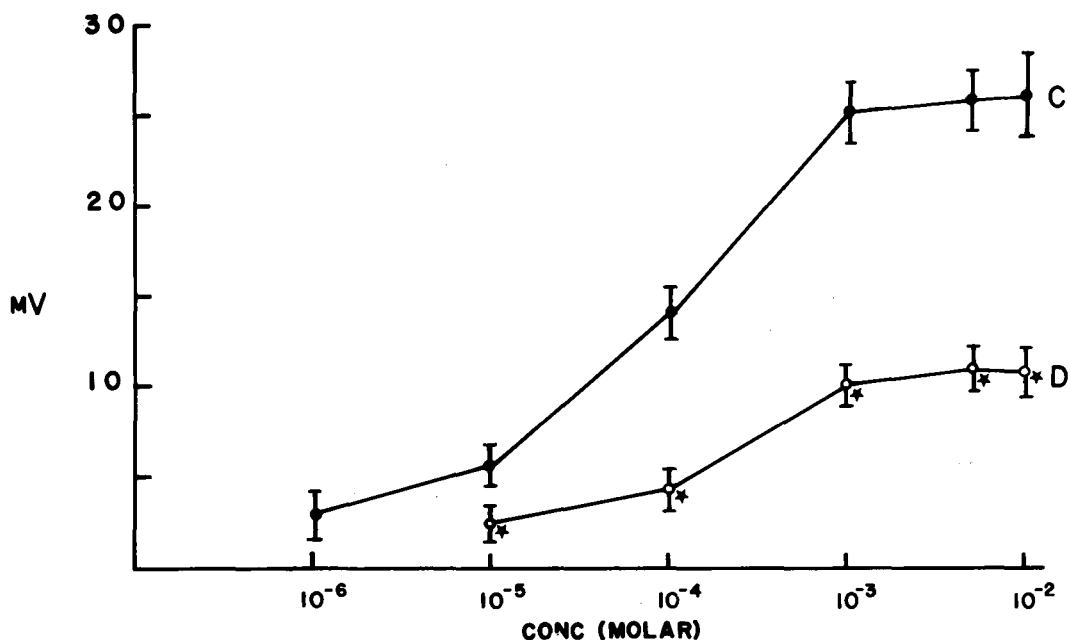


Figure 16. The dose-response curves of nicotinic responses to ACh on control and denervated ganglion cells in the presence of atropine ( $1 \times 10^{-5}$  M). Ordinate, depolarization in mV; abscissa, molar concentration of ACh. Each point on the curves represents the mean of results from eight ganglion cells. Vertical bars indicate S.E.M. Asterisk denotes statistically significant difference from control ( $P < 0.05$ ) using unpaired Student's t-test.

after denervation.

2. ACh responses on curarized control and denervated ganglion cells

As stated before, the curarized preparation was used to measure muscarinic response of ACh.

In control ganglion cells, ACh ( $1 \times 10^{-6} \text{M}$ ) did not produce any detectable depolarizing or hyperpolarizing potential. The minimum concentration of ACh that induced a detectable depolarization was  $1 \times 10^{-5} \text{M}$ . At the concentration of ACh ( $1 \times 10^{-3} \text{M}$ ), a noticeable depolarization was detected 40 sec after the start of ACh application (Fig. 17), nearly four times longer than needed for nicotinic response. At the peak of muscarinic depolarization, direct intracellular stimulation failed to elicit any action potential. Direct spikes regained their full amplitude after the membrane potential had returned to its resting level.

In about 10% of the control ganglion cells examined, a high concentration of ACh ( $1 \times 10^{-3} \text{M}$ ) failed to elicit any detectable muscarinic depolarization. It appears that not all cells are endowed with muscarinic receptors or the receptors are present in such a small area that the response to extrinsically applied ACh becomes undetectable.

The muscarinic depolarization was not accompanied by a decrease in membrane resistance as measured from the amplitude of repetitively induced electrotonic potentials; often a slight increase in membrane resistance was noted.

On denervated ganglion cells, ACh in concentration above  $1 \times 10^{-5} \text{M}$  consistently produced a depolarizing response



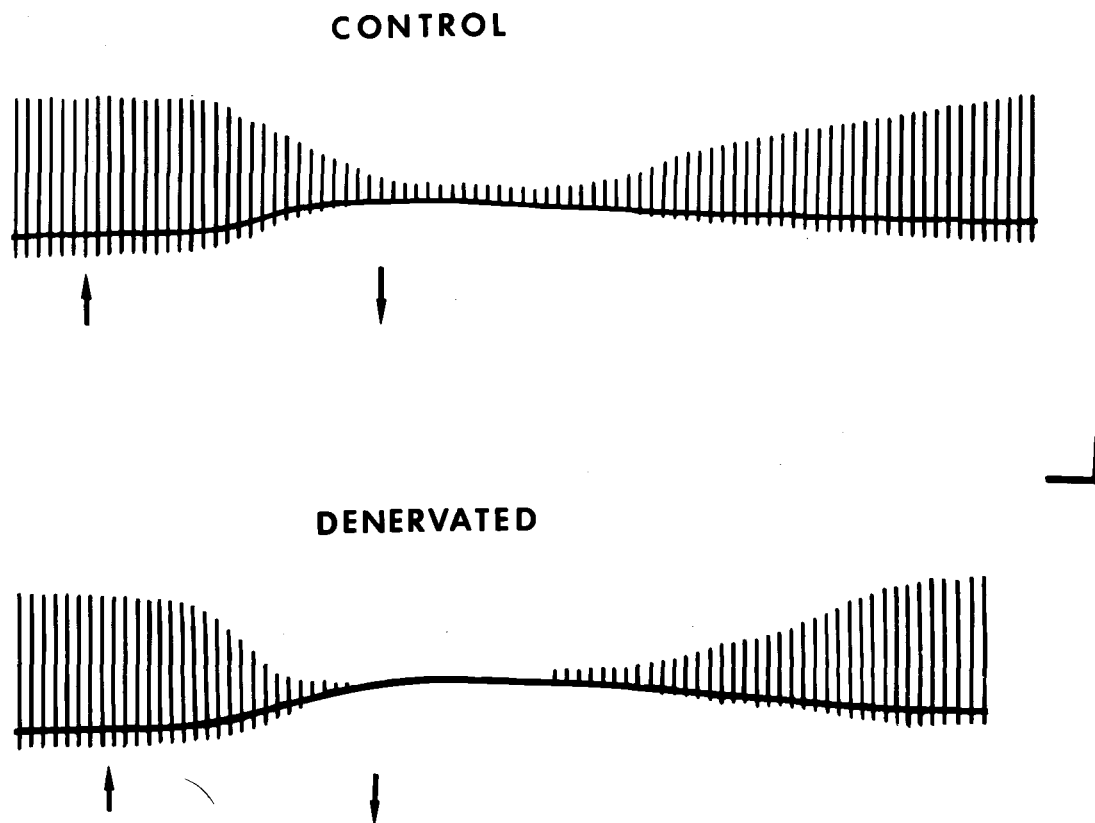


Figure 17. Intracellular recordings of the effect of ACh ( $1 \times 10^{-3}$  M) on a control (upper tracing) and a denervated (lower tracing) ganglion cell in the presence of d-Tc ( $5 \times 10^{-5}$  M). Action potentials were elicited by direct intracellular stimulation. Arrows indicate the start and withdrawal of ACh perfusion. Calibration: 20 mV and 10 sec.

which was not preceded by a hyperpolarizing response.

Fig. 17 demonstrates the effect of ACh ( $1 \times 10^{-3} \text{M}$ ) on a denervated ganglion cell. The depolarization developed earlier and the maximal amplitude was nearly double the control response. Action potentials induced by direct intracellular stimulation were completely eliminated at the plateau of depolarization and did not regain their full amplitude until membrane potential had repolarized to its resting level.

The dose-response curves of muscarinic depolarization in both control and denervated ganglion cells exhibited a characteristic S-shape (Fig. 18). The average response in denervated cells was about 50% greater than the control response; at the concentration of  $1 \times 10^{-3} \text{M}$  the difference was close to 80%.

### 3. Carbachol responses on atropinized control and denervated ganglion cells

In most respects, nicotinic depolarization induced by Carb was similar to that produced by nicotinic action ACh. One most noticeable difference was that the duration of nicotinic depolarization induced by Carb was prolonged in control as well as in denervated ganglion cells.

The dose-response curves of nicotinic action of Carb on control and denervated ganglion cells are illustrated in Fig. 19. The minimum effective concentration was elevated 100 fold and the maximal depolarization decreased nearly 60% after denervation.

## ACh RESPONSES ON CURARIZED CONTROL AND DENERVATED GANGLION CELLS

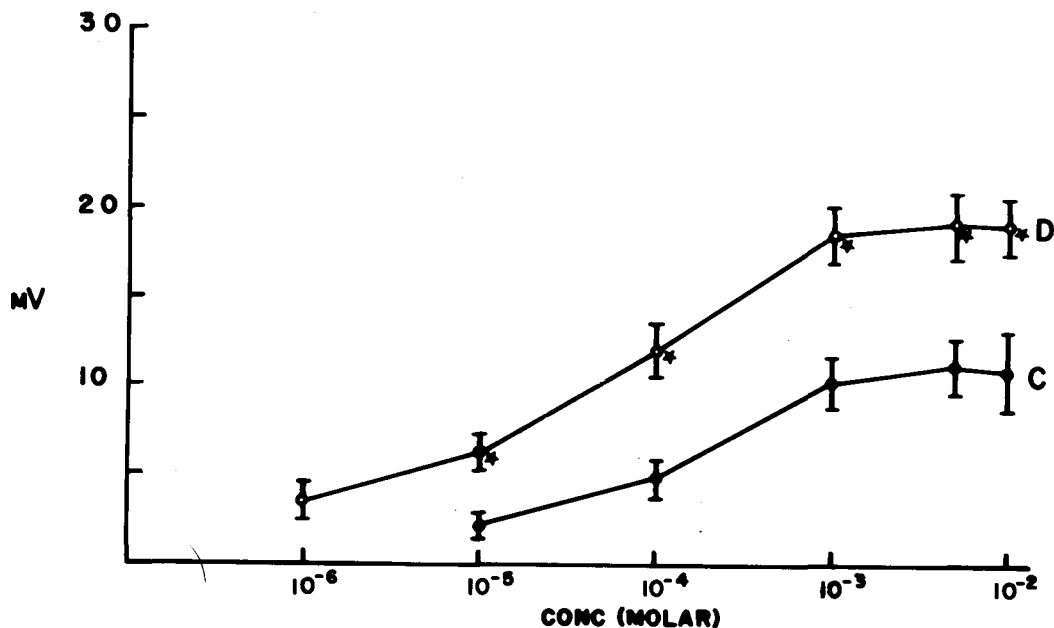


Figure 18. The dose-response curves of muscarinic responses to ACh on control and denervated ganglion cells in the presence of d-Tc ( $5 \times 10^{-5}$  M). Ordinate, depolarization in mV; abscissa, molar concentration of ACh. Each point on the curves represents the mean of results from six ganglion cells. Vertical bars indicate S.E.M. Asterisk denotes statistically significant difference from control ( $P < 0.05$ ) using unpaired Student's t-test.

## CARB RESPONSES ON ATROPINIZED CONTROL AND DENERVATED GANGLION CELLS

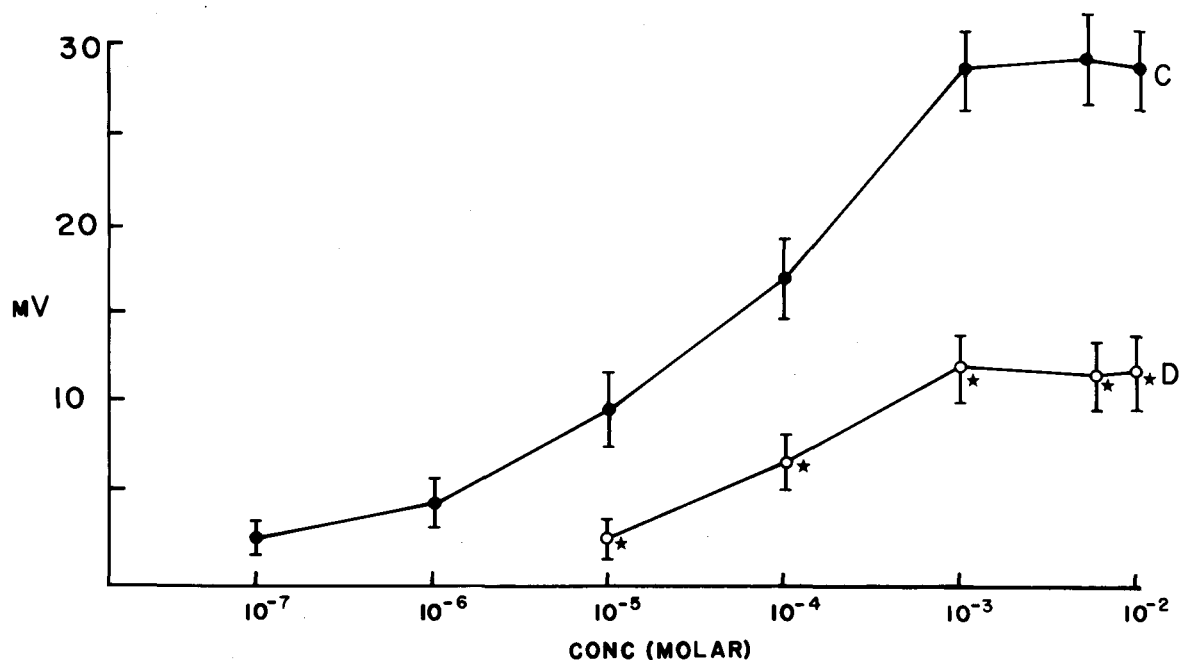


Figure 19. The dose-response curves of nicotinic responses to Carb on control and denervated ganglion cells in the presence of atropine ( $1 \times 10^{-5}$  M). Ordinate, depolarization in mV; abscissa, molar concentration of Carb. Each point on the curves represents the mean of results from seven ganglion cells. Vertical bars indicate S.E.M. Asterisk denotes statistically significant difference from control ( $P < 0.05$ ) using unpaired Student's t-test.

4. Carbachol responses on curarized control and denervated ganglion cells

Fig. 20 demonstrates the dose-response curves of muscarinic action of Carb on control and denervated ganglion cells. The muscarinic depolarization was enhanced in denervated cells as evident in the lower minimum effective concentration and higher amplitude. The dose-response curve shows a parallel shift to the left after denervation.

5. Methacholine responses on control and denervated ganglion cells

Fig. 21 illustrates the effect of MeCh ( $1 \times 10^{-3} \text{M}$ ) on a control ganglion cell. The muscarinic depolarization developed gradually and was not preceded by any visible hyperpolarizing potential. Upon the withdrawal of MeCh perfusion, orthodromic spikes regained their full amplitude shortly after membrane potential had returned to its resting level. No after-hyperpolarization was noted after removing MeCh from perfusing solution.

The effect of MeCh ( $1 \times 10^{-3} \text{M}$ ) on a denervated ganglion cell is depicted in Fig. 21. The membrane depolarization started earlier and reached the peak nearly twice as fast as the control cell response. At the peak of depolarization, spikes induced by direct intracellular stimulation were completely abolished. The amplitude of maximal depolarization was about 40% higher than the control. The rate of repolarization was slower and direct spikes regained their full amplitude after the membrane potential had returned to its initial level.

## CARB RESPONSES ON CURARIZED CONTROL AND DENERVATED GANGLION CELLS

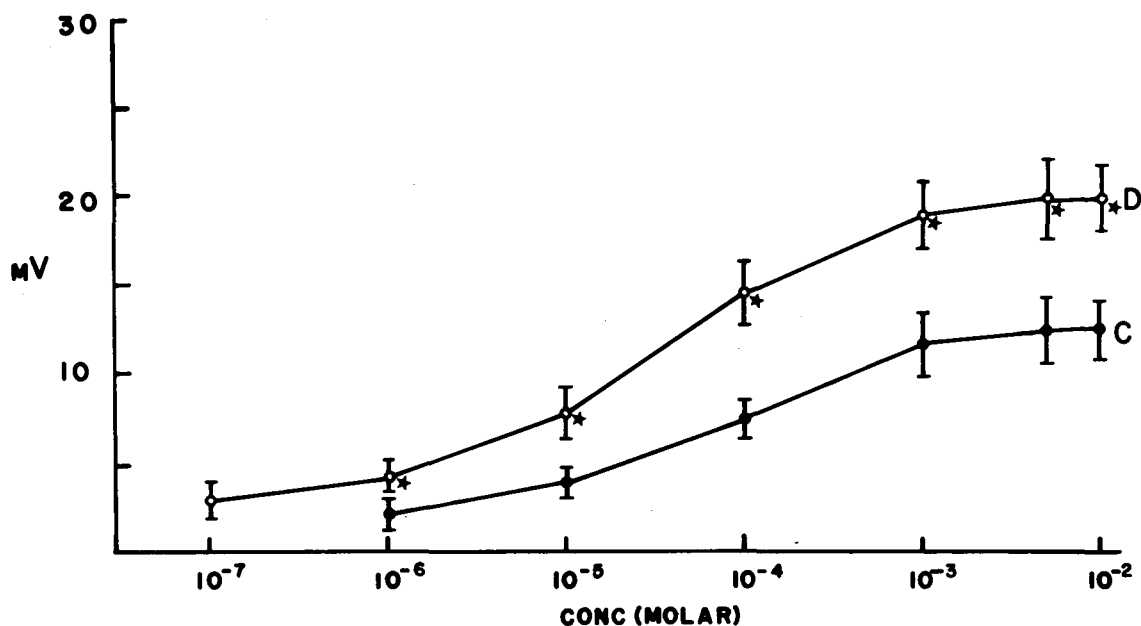


Figure 20. The dose-response curves of muscarinic responses to Carb on control and denervated ganglion cells in the presence of d-Tc ( $5 \times 10^{-5}$  M). Ordinate, depolarization in mV; abscissa, molar concentration of Carb. Each point on the curves represents the mean of results from five ganglion cells. Vertical bars indicate S.E.M. Asterisk denotes statistically significant difference from control ( $P < 0.05$ ) using unpaired Student's t-test.

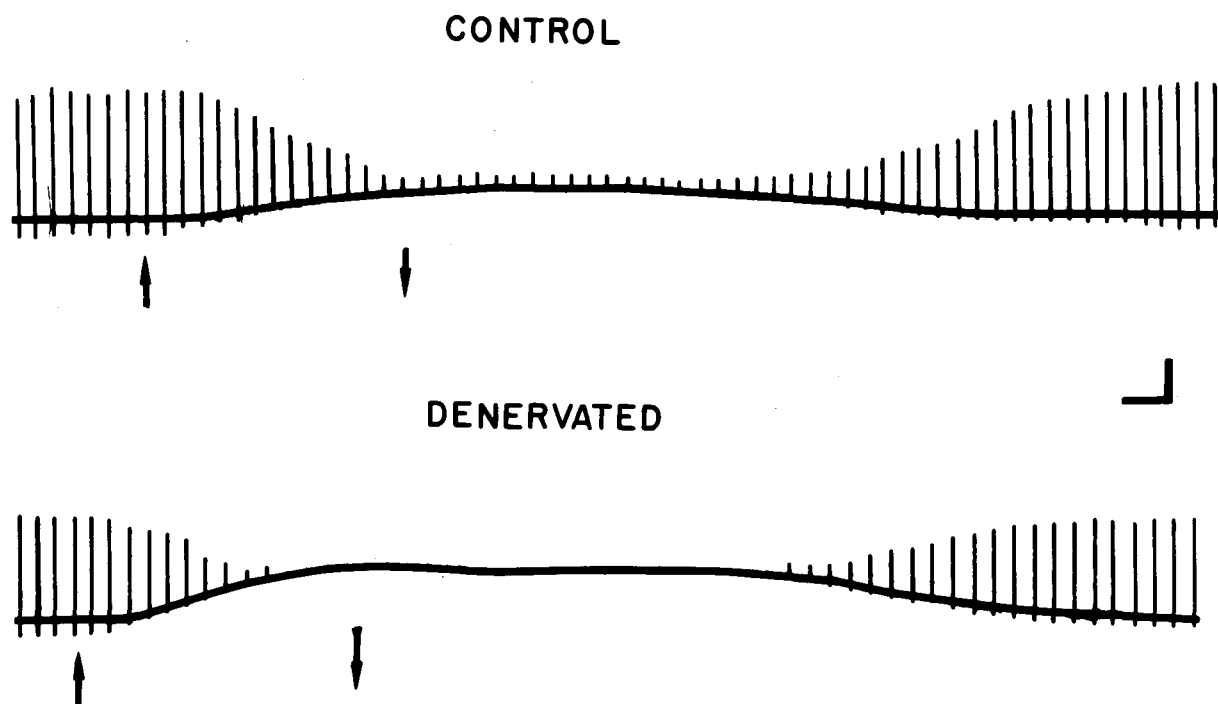


Figure 21. Intracellular recordings of the effect of MeCh ( $1 \times 10^{-3}$  M) on a control (upper tracing) and a denervated (lower tracing) ganglion cell. Action potentials were elicited by direct intracellular stimulation. Arrows indicate the start and withdrawal of MeCh perfusion. Calibration: 20 mV and 10 sec.

The depolarizing action of MeCh on control and denervated ganglion cells could be totally prevented by atropine ( $1 \times 10^{-5}$  M) and was not affected by d-Tc ( $5 \times 10^{-5}$  M).

The dose-response curves of MeCh on control and denervated ganglion cells are shown in Fig. 22. After denervation, the amplitude of MeCh induced muscarinic depolarization was increased and the difference was statistically significant at each concentration level.

#### 6. Effects of dopamine on control and denervated ganglion cells

Dopamine ( $1 \times 10^{-4}$  M) when added to the perfusion solution caused a consistent decrease in the amplitude of the e.p.s.p. in all 15 cells studied. A typical recording is shown in Fig. 23. The average decrease of e.p.s.p. amplitude in 15 cells 2 min after dopamine ( $1 \times 10^{-4}$  M) perfusion was  $34 \pm 4.8\%$  (mean  $\pm$  S.E.M.). Dopamine ( $1 \times 10^{-4}$  M) reduced the orthodromic action potential to a subthreshold e.p.s.p. (Fig. 24A). In many cells, impulse transmission could be temporarily restored by increasing the stimulus frequency (Fig. 24B). Dopamine ( $1 \times 10^{-3}$  M) completely blocked synaptic transmission in less than 2 min.

Dopamine ( $1 \times 10^{-4}$  M) induced a slight (2-5 mV) postsynaptic hyperpolarization without any accompanying alteration in membrane conductance. The postsynaptic membrane sensitivity to ACh applied iontophoretically was not affected by dopamine (Fig. 25).



## MeCh RESPONSES ON CONTROL AND DENERVATED GANGLION CELLS

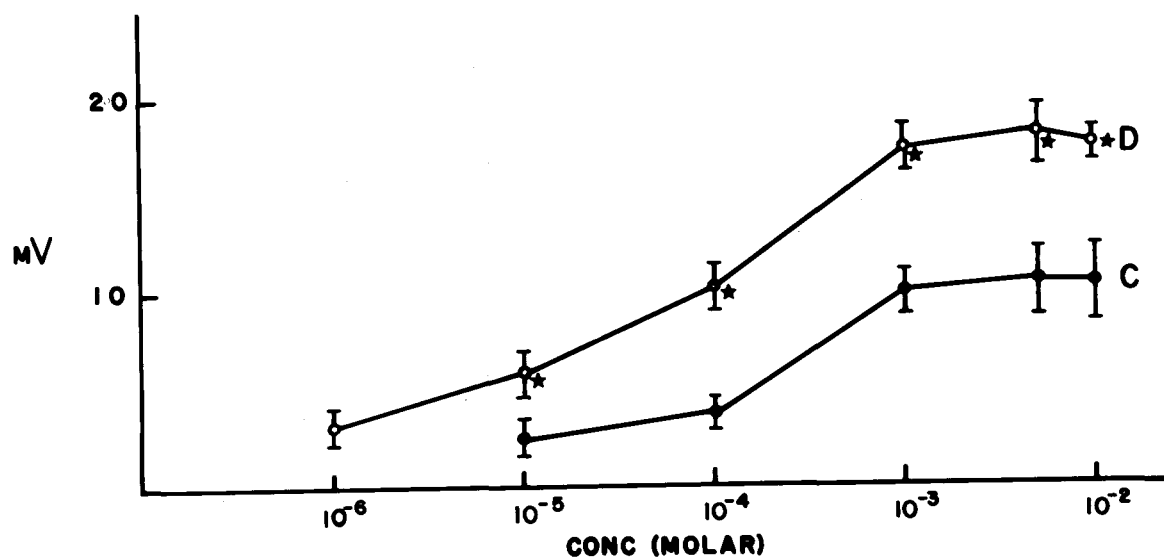


Figure 22. The dose-response curves of muscarinic responses to MeCh on control and denervated ganglion cells. Ordinate, depolarization in mV; abscissa, molar concentration of MeCh. Each point on the curves represents the mean of results from five ganglion cells. Vertical bars indicate S.E.M. Asterisk denotes statistically significant difference from control ( $P < 0.05$ ) using unpaired Student's t-test.

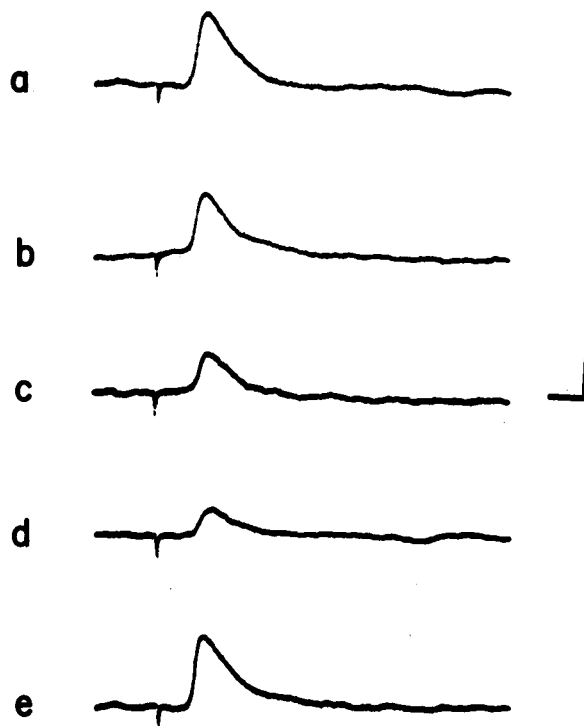


Figure 23. Effect of dopamine ( $1 \times 10^{-4}$  M) on e.p.s.p. of a single cell. a: control, b, c and d: 40sec, 100 sec and 120 sec after beginning of dopamine perfusion, respectively, e: 240 sec after washout with drug-free solution. Calibration: 10 mV and 10 msec.

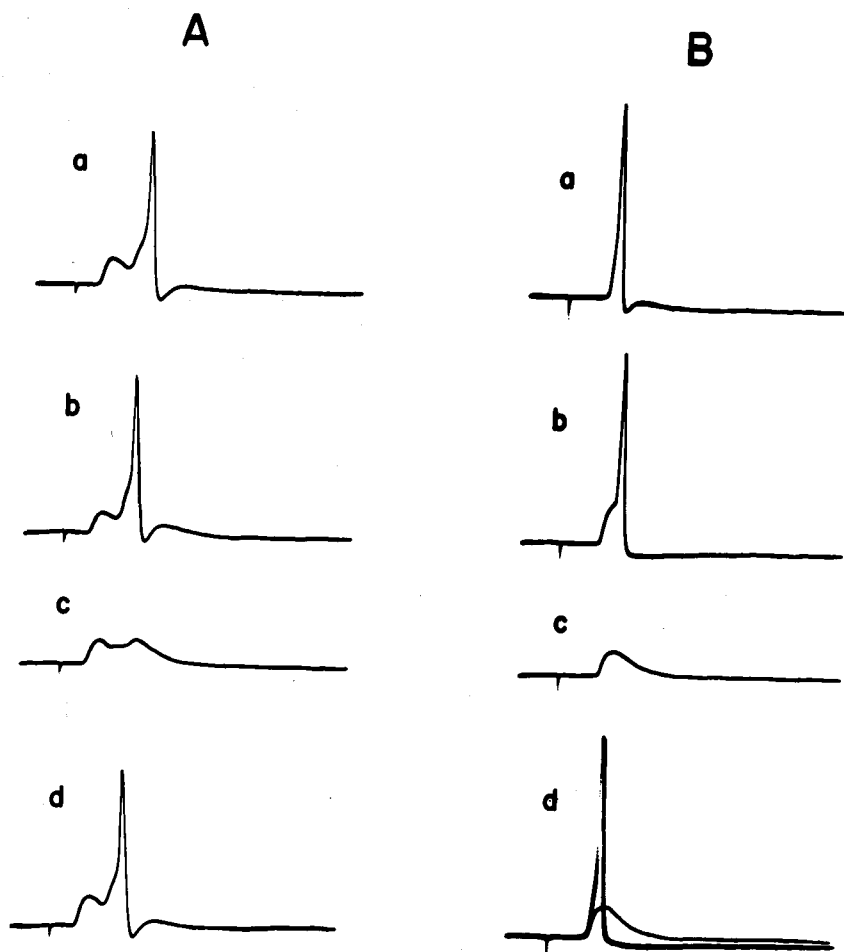


Figure 24. Effect of dopamine ( $1 \times 10^{-4}$  M) on orthodromic action potential. A: a, control; b and c, 60 sec and 160 sec after beginning of dopamine perfusion, respectively; d, 240 sec after washout with drug-free solution. B: a, control; b, c and d, 60 sec, 120 sec and 130 sec after beginning of dopamine perfusion, respectively. Notice in d, a reversal of transmission block by increasing stimulus frequency from 1 to 30 Hz. Records A and B were taken from two different cells. Calibration: 10 mV and 10 msec.

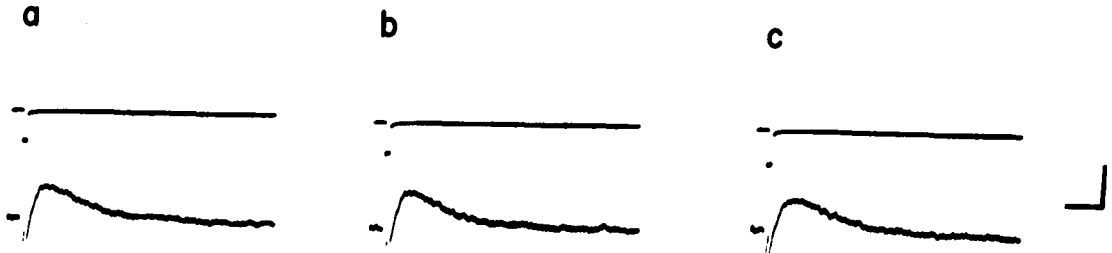


Figure 25. Effect of dopamine ( $1 \times 10^{-4}$  M) on ACh-potential. a: control, b: 120 sec after addition of dopamine, c: 120 sec after washout with drug-free solution. ACh was injected iontophoretically from a microelectrode by a current pulse of  $6.7 \times 10^{-8}$  A (upper traces). Calibration: 10 mV and 50 msec.

Dopamine ( $1 \times 10^{-4} \text{M}$ ) decreased the frequency of m.e.p.s.p.s with little change in the amplitude of m.e.p.s.p.s in seven cells studied; the average per cent decrease was  $31.7 \pm 8.1$  (mean  $\pm$  S.E.M.). Dopamine ( $1 \times 10^{-4} \text{M}$ ) reduced the quantal content of e.p.s.p. in a low Ca (0.2 mM) and high Mg (5.5 mM) solution, but had no effect on the quantal size (Dun & Nishi, 1974).

The ganglionic blocking effect of dopamine was antagonized by pretreating the ganglion with phenoxybenzamine ( $1 \times 10^{-5} \text{M}$ ) but was not antagonized by propranolol ( $5 \times 10^{-5} \text{M}$ ).

The results altogether show that the ganglionic depressant effect of dopamine is exerted primarily through an alpha-adrenoceptive site at the presynaptic nerve terminals.

In denervated ganglion cells, dopamine ( $1 \times 10^{-4} \text{M}$ ) elicited a larger hyperpolarizing potential (3-7 mV). Five of the 15 control ganglion cells failed to respond to the hyperpolarizing action of dopamine ( $1 \times 10^{-4} \text{M}$ ), whereas only two out of eleven denervated ganglion cells did not show any detectable hyperpolarizing response to the same concentration of dopamine. There is, thus, a slight increase in the sensitivity of ganglion cells to the hyperpolarizing action of dopamine after denervation. Pretreating the control and denervated ganglion cells with phenoxybenzamine ( $1 \times 10^{-5} \text{M}$ ) for 15 to 20 min completely obliterated the hyperpolarizing potential induced by dopamine.

## CHAPTER III. RESULTS

### C. PASSIVE AND ACTIVE MEMBRANE PROPERTIES

Electrical membrane properties were studied on 52 control ganglion cells taken from ten control ganglia and 50 denervated ganglion cells taken from 12 denervated ganglia.

#### 1. Resting membrane properties

When a cell was satisfactorily penetrated by a microelectrode, the resting membrane potential could be well maintained over 30 min. The mean resting membrane potential values of control and denervated ganglion cells were  $-57.9 \pm 1.5$  mV and  $-58.8 \pm 1.1$  mV (S.E.M.), respectively.

To determine the membrane time constant and membrane resistance of the resting membrane, anodal current pulses of increasing intensity were applied through the microelectrode to displace the membrane potential artificially. The membrane potential increased linearly with the intensity of current pulses applied. However, deviation from linearity became apparent when the cell membrane was artificially displaced over 50 mV from the resting level. This anomalous rectification is a characteristic of sympathetic neurons (Christ & Nishi, 1973). Fig. 26 depicts two sets of recordings of anelectrotonic potentials obtained from a control and a denervated ganglion cell. To the left of the recordings are graphic representation of current and voltage relation-

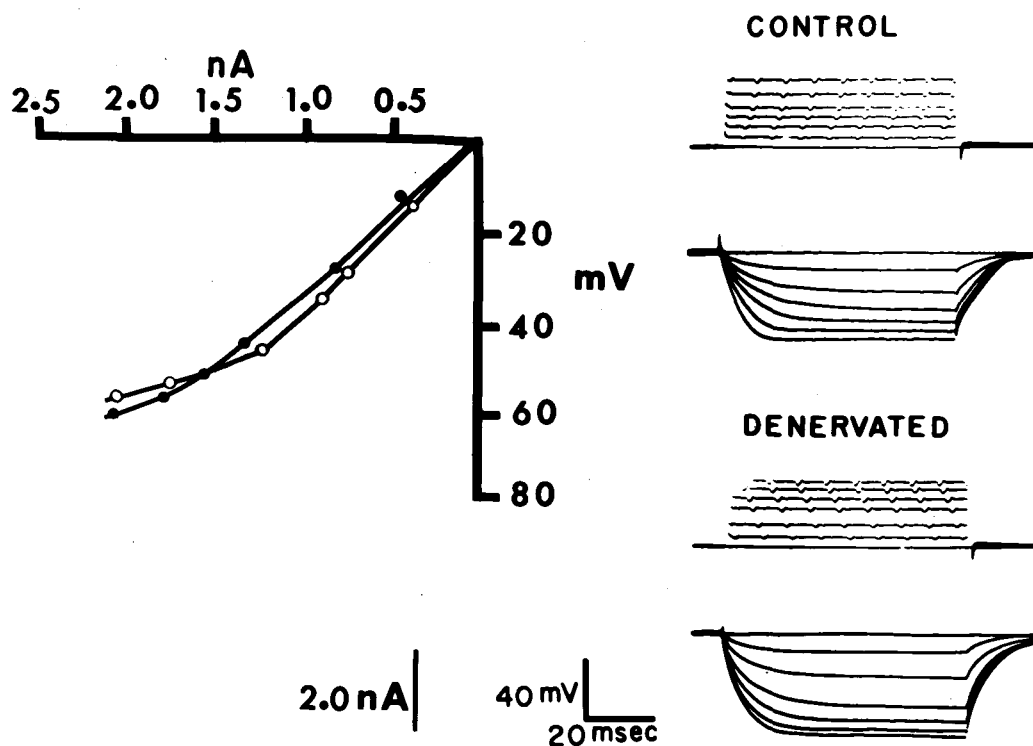


Figure 26. Anelectrotonic potentials recorded from a control ganglion cell (upper pair of tracings) and a denervated ganglion cell (lower pair of tracings). Upper tracing of each pair is the current pulses. To the left are V-I relationships; the solid circles represent the control responses and the open circles represent the denervated responses. Ordinate, potentials in mV; abscissa, current pulse intensities in  $\eta$ A.

ships. In both control and denervated ganglion cells, deviation of linearity of the V-I relationship occurred at a membrane potential of about 50 mV more negative than resting membrane potential. The effective membrane resistance was calculated from the linear portion of the V-I relationship. The mean values of effective membrane resistance before and after denervation were  $36.7 \pm 1.0 \text{ M}\Omega$  and  $37.0 \pm 1.3 \text{ M}\Omega$  (mean  $\pm$  S.E.M.), respectively. The mean values of membrane time constant in control and denervated ganglion cells were  $14.0 \pm 0.3 \text{ msec}$  and  $13.4 \pm 0.2 \text{ msec}$  (mean  $\pm$  S.E.M.), respectively. Thus, no statistically significant differences were found in the passive membrane characteristics between the control and denervated ganglion cells.

## 2. Active membrane properties

Typical recordings of an action potential elicited by direct intracellular stimulation from a control and a denervated ganglion cell are shown in Fig. 27. The configuration of the action potential remained the same before and after denervation. Table I summarizes the values of various parameters of the action potentials measured from 52 control ganglion cells and 50 denervated ganglion cells. Statistical analysis showed that all these parameters remained unchanged after denervation.



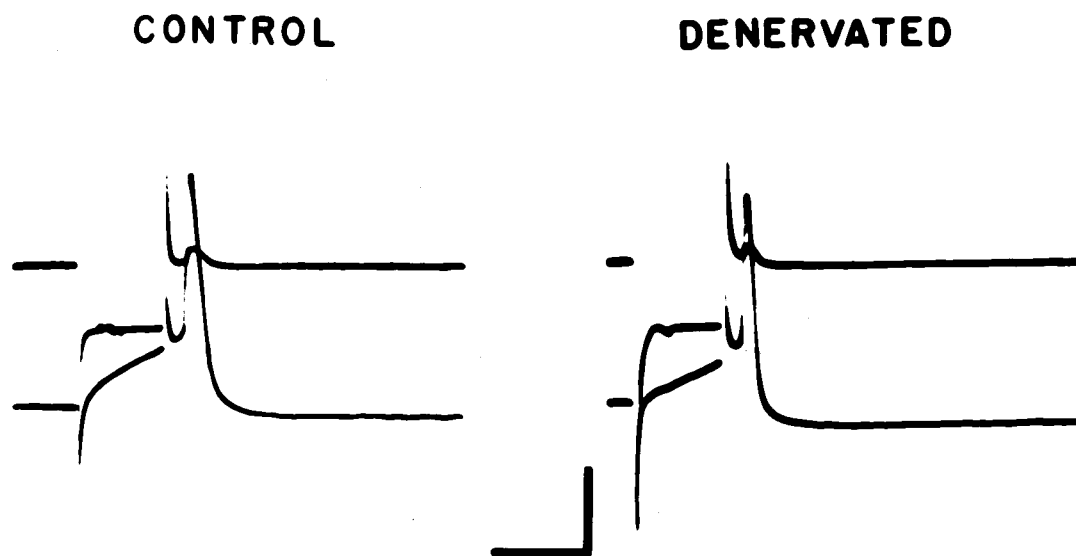


Figure 27. Recordings of action potentials elicited by direct intracellular stimulation from a control and a denervated ganglion cell. Calibration: 40 mV and 10 msec.

Table 1. ACTIVE MEMBRANE PROPERTIES OF CONTROL AND DENERVATED GANGLION CELLS\*

	Control		Denervated	
	Range	Mean $\pm$ S.E.M.	Range	Mean $\pm$ S.E.M.
Threshold Potential (mV)	-25 - -38	-31.8 $\pm$ 0.48	-24 - -38	-30.9 $\pm$ 0.53
Spike Amplitude (mV)	62 - 94	71.8 $\pm$ 0.91	59 - 97	70.4 $\pm$ 1.08
Time to Peak (msec)	1.2 - 2.7	1.75 $\pm$ 0.05	1.3 - 2.5	1.77 $\pm$ 0.04
Spike Total Duration (msec)	3.3 - 5.7	4.77 $\pm$ 0.08	3.6 - 5.8	4.82 $\pm$ 0.05
Maximum Rate of Rise (V/sec)	265 - 330	303.6 $\pm$ 2.27	275 - 335	299.0 $\pm$ 2.06
Maximum Rate of Fall (V/sec)	64 - 88	70.7 $\pm$ 0.59	62 - 85	69.6 $\pm$ 0.50
Amplitude of AH <sup>†</sup> (mV)	12 - 26	15.6 $\pm$ 0.56	10 - 29	16.4 $\pm$ 0.48
Half Decay Time of AH (msec)	35 - 58	44.3 $\pm$ 1.78	31 - 61	42.6 $\pm$ 1.90
Reversal Potential of AH (mV)	-86 - -98	-92.6 $\pm$ 2.45	-84 - -96	-93.7 $\pm$ 3.12

\* Results were obtained from 52 control and 50 denervated ganglion cells.

† After-hyperpolarization of an action potential.

## CHAPTER III. RESULTS

### C. EQUILIBRIUM POTENTIAL OF THE SUBSYNAPTIC MEMBRANE ( $E_{\text{epsp}}$ )

ACh was applied iontophoretically to 18 control ganglion cells of which 12 cells showed a single nicotinic response which was not followed by any slow depolarizing or hyperpolarizing response sensitive to the blocking action of atropine. The nicotinic response was completely and reversibly blocked by d-Tc in concentration of  $5 \times 10^{-5} \text{M}$ . At the membrane potential of approximately  $-50 \text{ mV}$ , the ACh induced nicotinic response showed a mean amplitude of  $14.6 \pm 3.5 \text{ mV}$  (mean  $\pm$  S.E.M.), a rise time of  $210 \pm 32 \text{ msec}$  (mean  $\pm$  S.E.M.) and a half-decay time of  $645 \pm 72 \text{ msec}$  (mean  $\pm$  S.E.M.). In six other cells, the induced ACh potential consisted of an initial nicotinic response which was followed by a small (2-5 mV) and slow (5-20 sec in total duration) muscarinic depolarization. Atropine ( $1 \times 10^{-5} \text{M}$ ) selectively blocked this slow depolarization. No hyperpolarizing response could be detected in these control cells.

Fig. 28 depicts the graphic representation of the equilibrium potential of three control ganglion cells by plotting the amplitude of ACh potential against the membrane potential. The X intercept of each line represents the  $E_{\text{epsp}}$ . The recordings on the left hand column are ACh

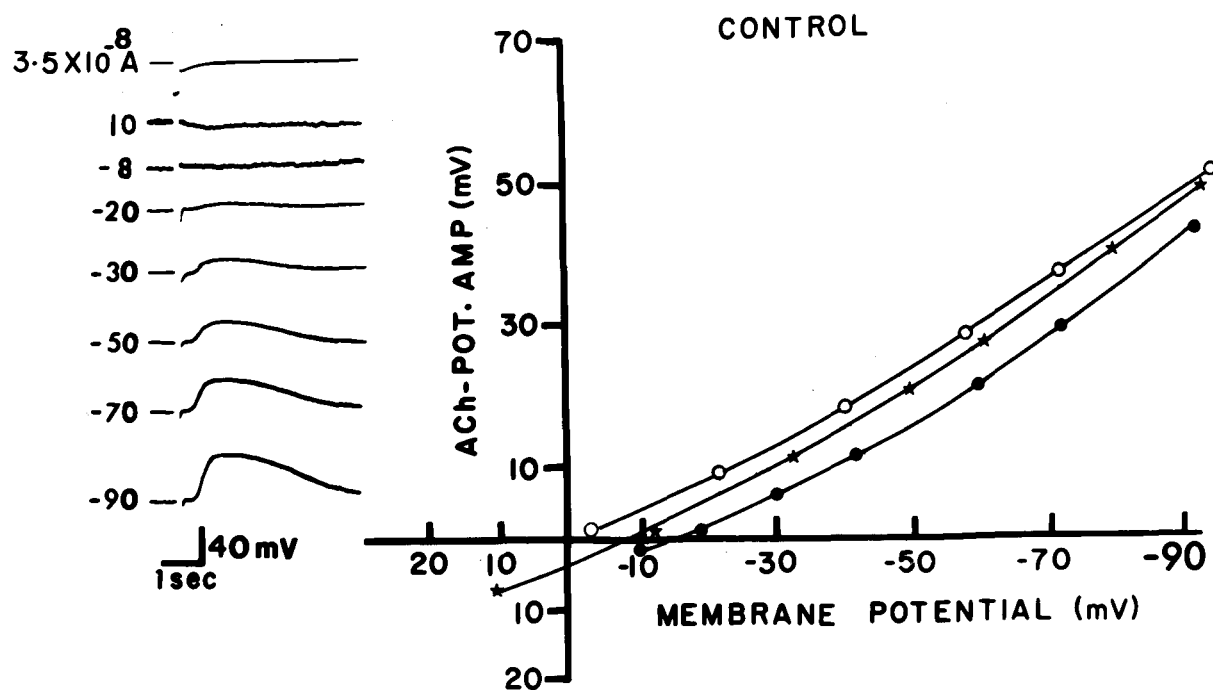


Figure 28. Equilibrium potential determination by iontophoresis of ACh from 3 different control ganglion cells. Ordinate, ACh-potential amplitude in mV; abscissa, membrane potential in mV. To the left are ACh-potential recordings obtained from a single control ganglion cell. The ACh-potential was nullified at the membrane potential of -8 mV which is taken as the equilibrium potential. Top tracing is the current pulse ( $3.5 \times 10^{-8}$  A) used to apply ACh iontophoretically.

potentials obtained from a single cell at various membrane potentials. The  $E_{\text{epsp}}$  in 18 control cells ranged between +2 mV and -20 mV and the mean value was  $-12.3 \pm 1.9$  (mean  $\pm$  S.E.M.). The addition of atropine ( $1 \times 10^{-6}$  -  $1 \times 10^{-5}$  M) did not alter the equilibrium potential of the subsynaptic membrane.

The mean amplitude of nicotinic ACh potential in 14 denervated ganglion cells was  $11.5 \pm 4.6$  mV (mean  $\pm$  S.E.M.) at the membrane potential of about -50 mV, a rise time of  $232 \pm 27$  msec (mean  $\pm$  S.E.M.), and a half-decay time of  $633 \pm 85$  msec (mean  $\pm$  S.E.M.). In eight of the denervated ganglion cells, the initial nicotinic response was followed by a larger depolarization (3-8 mV) with a time course of about 5 to 32 sec. Again, atropine ( $1 \times 10^{-5}$  M) selectively and reversibly blocked the second slow depolarization.

The equilibrium potentials obtained from three denervated cells are shown in Fig. 29. ACh potentials at various membrane potentials from a single denervated cell can be seen in the left-hand column. In this particular experiment, atropine was not added. The fast nicotinic depolarization was followed by a noticeable hyperpolarizing potential. Attempt was made to elucidate the nature of this hyperpolarizing potential. It was found that this hyperpolarizing potential occurred at membrane potentials between -30 mV and -70 mV; further increase or decrease in membrane potential nullified this hyperpolarizing potential. In concentration of  $5 \times 10^{-5}$  M, d-Tc abolished the fast nicotinic de-

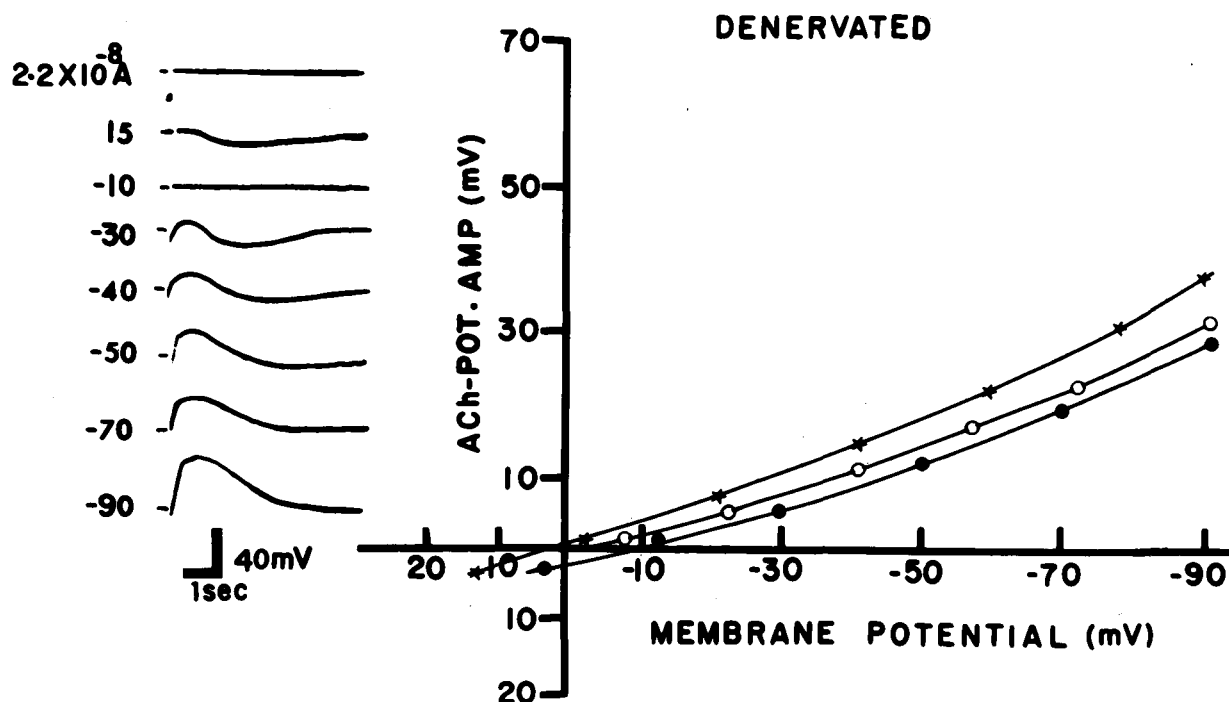


Figure 29. Equilibrium potential determination by iontophoresis of ACh from 3 different denervated ganglion cells. Ordinate, ACh-potential amplitude in mV; abscissa, membrane potential in mV. To the left are ACh-potential recordings obtained from a single denervated ganglion cell. The ACh-potential was nullified at the membrane potential of -10 mV which is taken as the equilibrium potential. Top tracing is the current pulse ( $2.2 \times 10^{-8}$  A) used to apply ACh iontophoretically.

polarizing potential but did not affect the amplitude of the hyperpolarizing potential; atropine ( $1 \times 10^{-5} \text{M}$ ) reversibly abolished the hyperpolarizing potential but had no effect on the depolarizing response (Fig. 30). Perfusing the ganglion with a low Ca and high Mg Krebs solution (Ca/Mg 0.1 mM/6.0 mM) gradually diminished this hyperpolarizing potential but did not attenuate the amplitude of the depolarizing response. Finally phenoxybenzamine ( $1 \times 10^{-5} \text{M}$ ) selectively depressed this hyperpolarizing potential. These results all together indicate that the hyperpolarizing potential is the slow i.p.s.p. generated by the stimulation of muscarinic site of chromaffin cells by injected ACh. This is in accordance with Libet's (1970) hypothesis of di-synaptic mediation of the slow i.p.s.p.

In the 14 denervated cells, the equilibrium potential of nicotinic ACh potential ranged between -6 and -23 mV. The mean equilibrium potential was  $13.2 \pm 1.3$  mV (mean  $\pm$  S.E.M.), which was slightly more negative than the control mean value. However, statistical analysis reveals that the difference is insignificant.

The ratio of change of Na conductance ( $\Delta g_{\text{Na}}$ ) and K conductance ( $\Delta g_{\text{K}}$ ) of the activated subsynaptic membrane was calculated employing Takeuchi and Takeuchi's (1960) equation. The calculated ratios of  $\Delta g_{\text{Na}}/\Delta g_{\text{K}}$  of the activated subsynaptic membrane of control and denervated ganglion cells are 1.68 and 1.72, respectively. The difference is not statistically significant.

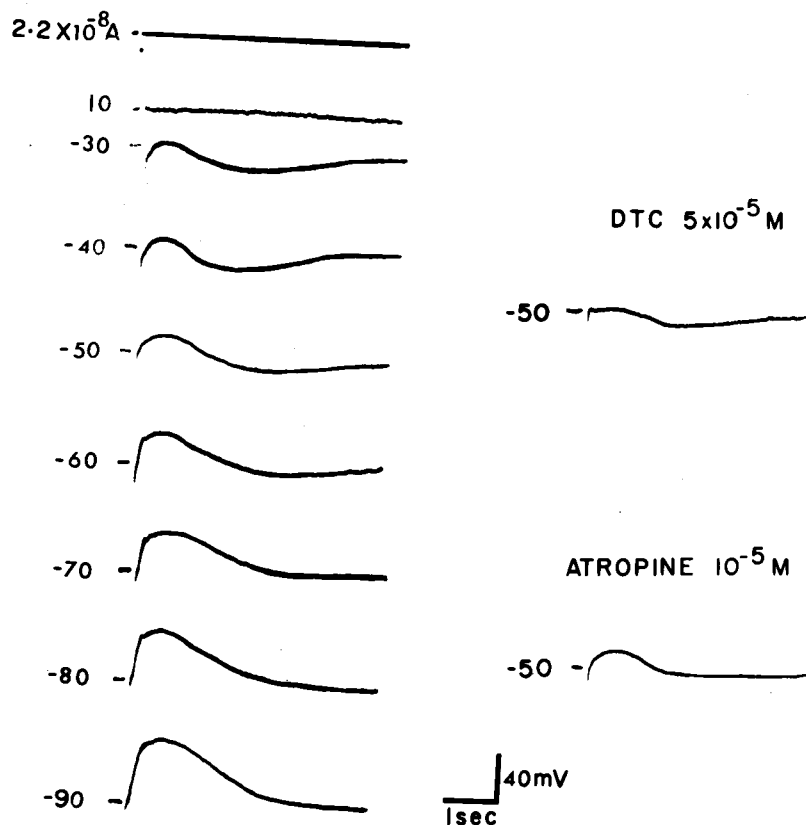


Figure 30. ACh-potential obtained from a single denervated ganglion cell. To the left are recordings of ACh-potential in normal Krebs solution, the fast ACh-potential is followed by a hyperpolarizing potential between the membrane potential -70 mV and -30 mV. To the right, the upper tracing is ACh-potential (membrane potential -50 mV) 3 min after the start of d-Tc ( $5 \times 10^{-5}$  M) perfusion; the lower tracing is ACh-potential (membrane potential -50 mV) 2 min after the beginning of atropine ( $1 \times 10^{-5}$  M) perfusion. ACh was injected iontophoretically by a current pulse of  $2.2 \times 10^{-8}$  A (top tracing of left column recordings).



## CHAPTER III. RESULTS

### E. DRUG-RECEPTOR INTERACTION

In analyzing the drug-receptor interaction, the double reciprocal plot was utilized to calculate  $K_a$ , the dissociation constant of drug-receptor complex. The  $K_a$  obtained from the intracellularly recorded ACh potentials was chosen as the prototype, since the results obtained from MeCh and Carb were essentially similar to ACh. The  $K_a$  extrapolated from the double reciprocal plots of atropinized control and denervated ganglion cell responses to ACh were  $9 \times 10^{-5}M$  and  $1.2 \times 10^{-4}M$ , respectively. The  $K_a$  extrapolated for curarized control and denervated ganglion cell responses were  $1.1 \times 10^{-4}M$  and  $1.0 \times 10^{-4}M$ , respectively.

The theoretical dose-response curves were constructed by assigning  $n = 1, 2, \dots, n$ ;  $n$  is the number of molecules of agonists that attached to a single receptor (cf. Takeuchi & Takeuchi, 1969). Fig. 31 demonstrates the experimental and theoretical dose-response curves of nicotinic action of ACh on control ganglion cells, on the assumption that  $n = 1$ . The theoretical curve corresponds reasonably well to the experimental curve. Deviation, however, did become apparent at the tail end of these two curves.

The experimental and theoretical curves of atropinized denervated ganglion cell responses to ACh are illustrated in

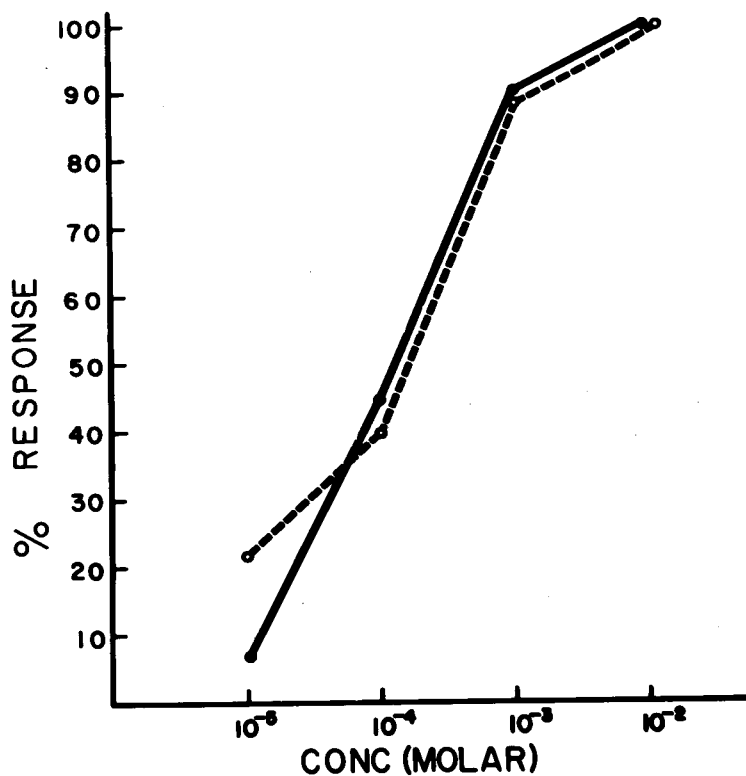


Figure 31. Theoretical and experimental curves of control ganglionic nicotinic responses of bath perfused ACh obtained from intracellular studies. Ordinate, percent response; abscissa, molar concentration of ACh. The theoretical curve (solid line) is constructed from the equation,  $y = 1 / (1 + K_a / A^n)$ , where  $n = 1$  (cf. Takeuchi and Takeuchi, 1969); the broken line represents the results obtained experimentally.

Fig. 32. The experimental and theoretical curves of curarized control and denervated ganglion cell responses to ACh are depicted in Fig. 33 and Fig. 34, respectively. In all these cases, the experimental plots fit closely to the theoretical plots.

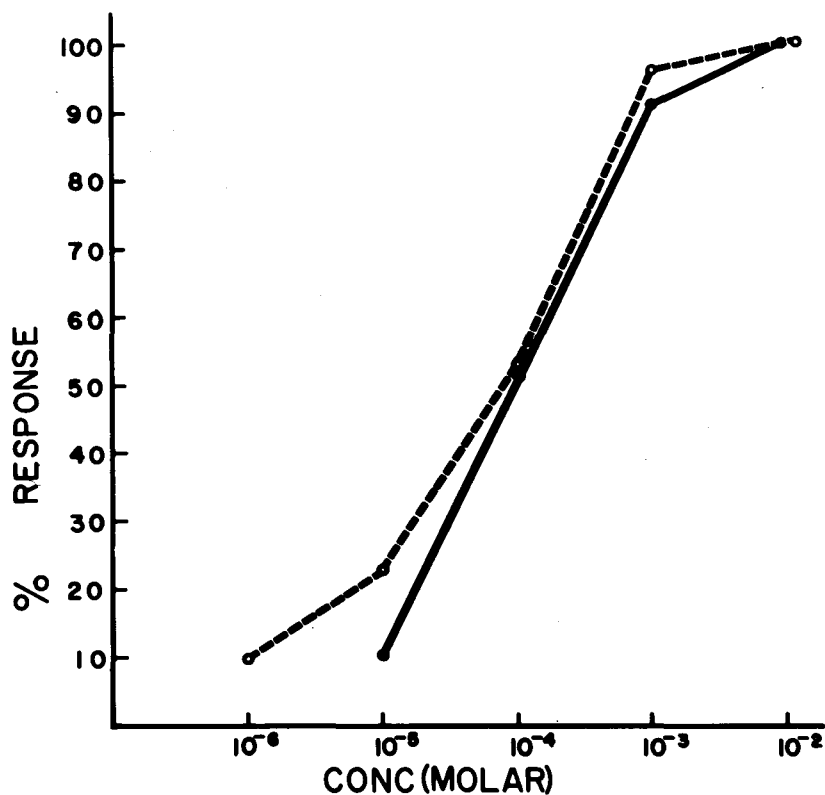


Figure 32. Theoretical and experimental curves of denervated ganglionic nicotinic responses to bath perfused ACh obtained from intracellular studies. Ordinate, percent responses; abscissa, molar concentration of ACh. The theoretical curve (solid line) is constructed from the equation,  $y = 1 / (1 + K_a / A^n)$ , where  $n = .1$  (cf. Takeuchi and Takeuchi, 1969); the broken line represents the results obtained experimentally.

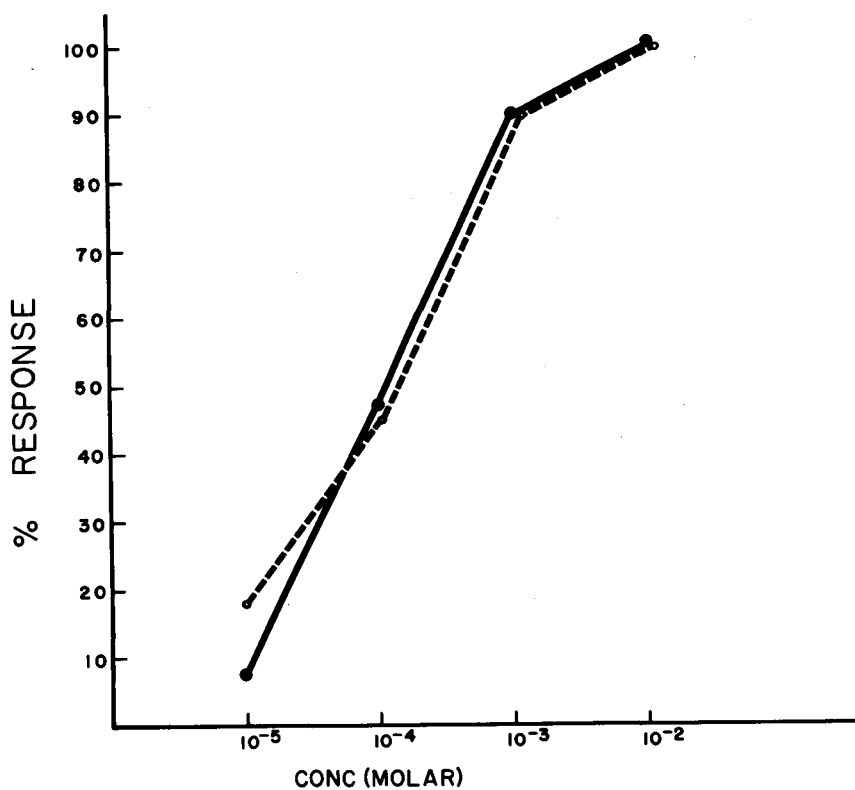


Figure 33. Theoretical and experimental curves of control ganglionic muscarinic responses to bath perfused ACh obtained from intracellular studies. Ordinate, percent response; abscissa, molar concentration of ACh. The theoretical curve (solid line) is constructed from the equation,  $y = 1 / (1 + K_a / A^n)$  where  $n = 1$  (cf. Takeuchi and Takeuchi, 1969); the broken line represents the results obtained experimentally.

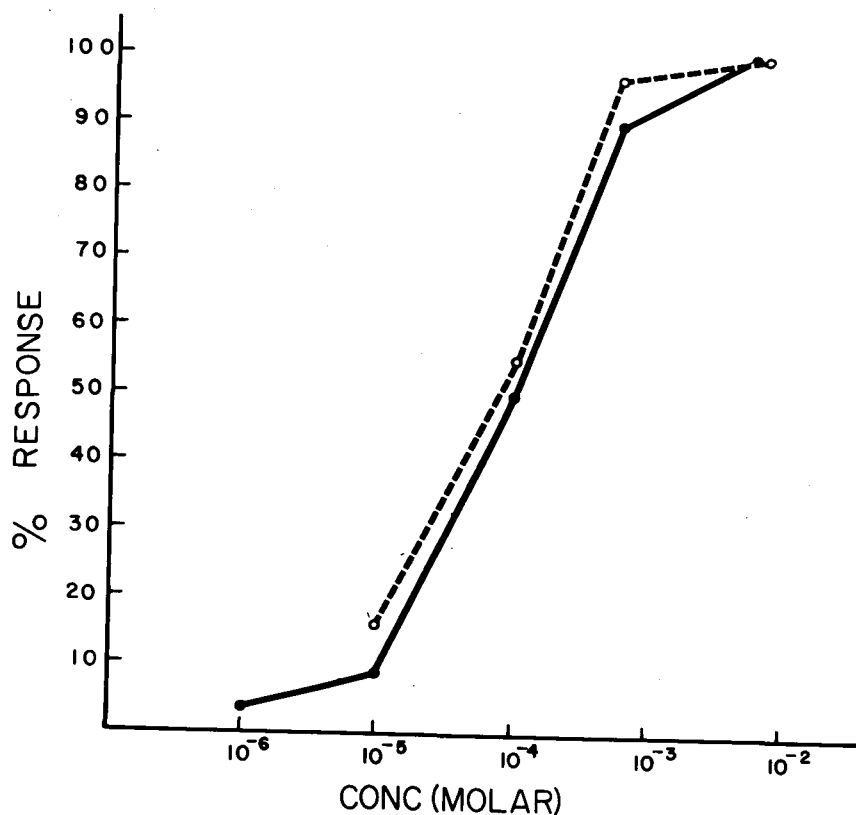


Figure 34. Theoretical and experimental curves of denervated ganglionic muscarinic responses to bath perfused ACh obtained from intracellular studies. Ordinate, percent response; abscissa, molar concentration of ACh. The theoretical curve (solid line) is constructed from the equation,  $y = 1 / (1 + K_a / A^n)$ , where  $n = 1$  (cf. Takeuchi and Takeuchi, 1969); the broken line represents the results obtained experimentally.

## CHAPTER III. RESULTS

### F. CELL DIAMETER MEASUREMENT

Measurement of cell diameter was made in three denervated ganglia and their contralateral unoperated ganglia. Cumulative soma diameter histograms are shown in Fig. 35. The cell diameter - mean of the major and minor axes distributed from about 15  $\mu\text{m}$  to 60  $\mu\text{m}$ . In each histogram, the mode was 25  $\mu\text{m}$ . The average cell diameters from 5350 control and 5335 denervated ganglion cells were 28.08  $\mu\text{m}$  and 28.45  $\mu\text{m}$ , respectively. There are two distinct peaks present in the cumulative histogram; one peak occurs at 25  $\mu\text{m}$  and the other peak is at 40 $\mu\text{m}$ .

## CELL DIAMETER HISTOGRAM

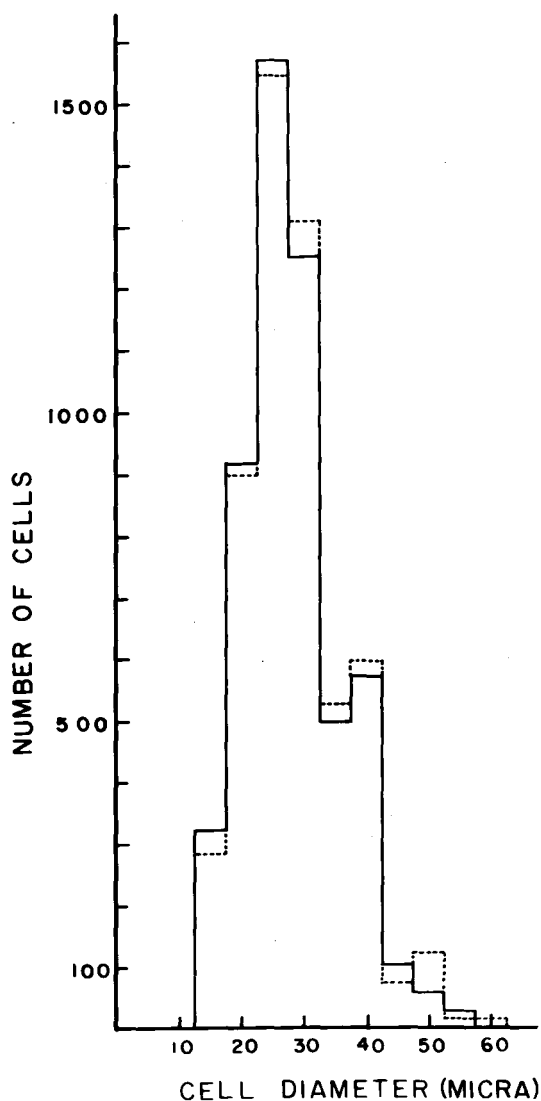


Figure 35. Cumulative histograms of control (solid line) and denervated (broken line) ganglion cell diameters obtained from 3 denervated ganglia and their contralateral unoperated ganglia. Ordinate, number of cells; abscissa, cell diameter in micra.



CHAPTER IV

DISCUSSION

## CHAPTER IV. DISCUSSION

The present study demonstrates that chronic preganglionic denervation alters the sensitivity of rabbit superior cervical ganglia to various cholinergic agents. Initial sucrose-gap experiments in which the entire ganglionic responses were measured provided evidence that nicotinic response to ACh or carbachol in the presence of atropine was depressed and the muscarinic response of ACh or carbachol in the presence of d-Tc was enhanced after denervation. The intracellular study in which nicotinic and muscarinic responses were obtained at the single cell level confirms these findings.

In early studies dealing with denervation phenomena, differentiation of nicotinic and muscarinic responses was not taken into consideration in the interpretation of data (Cannon & Rosenblueth, 1936; Chein, 1960; Volle & Koelle, 1961; Jones, 1963; Brown, 1969). In the present investigation, ganglionic nicotinic and muscarinic responses were studied independently by suppressing one response with a specific blocking agent while the other response was being investigated; atropine in a concentration of  $1 \times 10^{-5} \text{M}$  was used to suppress muscarinic responses and d-Tc ( $5 \times 10^{-5} \text{M}$ ) was employed to block nicotinic responses, respectively.

Atropine in concentrations between  $1 \times 10^{-6} \text{M}$  and  $1 \times 10^{-4} \text{M}$  has been shown to block specifically the ganglionic muscarin-

ic response of ACh (Eccles & Libet, 1961; Takeshige & Volle, 1962; Koketsu et al., 1968). Close iontophoretic application of ACh to frog sympathetic ganglion cells elicits two distinct components; the first one is a fast rising large depolarization (fast ACh potential) which is followed by a slow depolarization (slow ACh potential). Nicotine and d-Tc specifically block the fast ACh potential without affecting the slow ACh potential, whereas atropine selectively and reversibly abolishes the slow ACh potential leaving the fast ACh potential intact (Koketsu et al., 1968). However, iontophoretic application of ACh to mammalian sympathetic neurons elicited, instead of two distinct components as in amphibian sympathetic neurons, a fast ACh potential followed by a not so distinct slow ACh potential which in most instances appeared in the falling phase of the fast ACh potential (Nishi, 1970). Atropine ( $1 \times 10^{-5} \text{M}$ ) selectively abolished the slow ACh potential, thus shortening the time course of the falling phase of the fast ACh potential. The present study confirms this finding. Furthermore, denervated ganglion cells responded to iontophoretic application of ACh with a slightly diminished fast ACh potential and an augmented slow ACh potential. Atropine specifically blocked the enhanced slow ACh potential but did not alter the response of the fast ACh potential. This indicates that denervation did not modify the lack of sensitivity of nicotinic receptors to atropine. In contrast, Beranek and Vyskocil (1967) reported

that atropine is more effective than d-Tc in blocking the focally induced ACh potential in denervated skeletal muscle.

In the present study, d-Tc ( $5 \times 10^{-5} \text{M}$ ) was found to suppress selectively and reversibly the fast ACh potential, whereas the slow ACh potential remained unaffected. This confirms previous studies that ganglionic nicotinic action of ACh is selectively abolished by d-Tc without altering the muscarinic action of ACh (Takeshige & Volle, 1962; Koketsu et al., 1968; Libet, 1970).

#### Dose-Response Curves

In constructing the dose-response curves, the bath perfusion method was adopted in which a known concentration of drug-Krebs solution was applied to the ganglion, whereas in determination of the equilibrium potentials of the nicotinic subsynaptic membrane the method of iontophoresis ACh was employed. The amplitude of the ACh potential induced by iontophoresis is critically dependent upon two important factors: (1) the distance between the ACh micropipette and the receptor area, and (2) the amount of ACh reaching the receptor area. In order to compare the amplitude of ACh potentials in a valid manner, the ACh potentials must all have about the same peak time. The peak time of ACh potential is proportional to the distance between the tip of the micropipette and the receptor area; this relationship is expressed by the following equation (cf. Koketsu et al., 1968):

$$r^2 = 6kT$$

where  $r$  is the distance between the tip of the micropipette and the receptor area,  $K$  is the diffusion coefficient for ACh (approximately  $8 \times 10^{-6} \text{ cm}^2 \text{ sec}^{-1}$ ) and  $T$  is the peak time of the ACh potential. The dose-response curves could be constructed using the iontophoresis method and would be valid if all the ACh potentials obtained in the control and denervated preparations had the same peak time. However, it proved very difficult to obtain a sufficiently large number of ACh potentials having the same peak time.

In the case of equilibrium potential determination, the peak time of the response is not as important as the amount of ACh reaching the receptor area. The best available method in delivering a constant amount of ACh to the ganglion cell in an impulsive manner is the iontophoretic application of ACh. The bath perfusion method is not satisfactory for this purpose. Therefore, dose-response curves were obtained with the bath perfusion method and equilibrium potentials were obtained with iontophoresis of ACh.

#### Depression of Nicotinic Response after Denervation

The present results indicate that the nicotinic response of the rabbit superior cervical ganglion to nicotinic agents was depressed after denervation as evidenced by an elevated minimum effective concentration and a nearly 50 per cent decrease in the amplitude of depolarization by a given concentration of nicotinic agent. Furthermore, the hyperpolarization that followed the nicotinic depolarization was

greatly attenuated in the denervated ganglia. According to Brown et al. (1972), the amplitude of this hyperpolarization is proportional to the preceding nicotinic depolarization. A reduction of the hyperpolarization in denervated ganglia is also in support of the finding that the nicotinic response decreases after denervation.

The ionic mechanism of the hyperpolarization following removal of depolarizing agents from sympathetic ganglia will be discussed briefly. Brown et al. (1972) proposed that the ionic mechanism of the hyperpolarization following removal of depolarizing agents may be due to an electrogenic extrusion of Na ions which are accumulated in the ganglion during the preceding nicotinic depolarization. Lees and Wallis (1974) made an extensive study of the electrogenesis of hyperpolarization and reached essentially the same conclusion; namely, that the hyperpolarization is due to the activity of an electrogenic Na pump system.

Several previous studies on cat superior cervical ganglia have revealed a decrease in nicotinic response to cholinergic agents after denervation (Volle & Koelle, 1961; Bokri et al., 1963; Brown, 1969; Green, 1969; Vickerson & Varma, 1969). The threshold dose of carbachol required to elicit postganglionic discharges in postganglionic nerve trunks were elevated in denervated ganglia (Volle & Koelle, 1961). They suggested, on the basis of this latter observation, that the primary action of carbachol is to stimulate

the release of ACh from presynaptic nerve terminals. It is clear now that carbachol acts directly to depolarize the postsynaptic membrane (Ginsborg & Guerrero, 1964); furthermore, the nicotinic receptors in presynaptic nerve terminals are of little consequence in the process of transmitter liberation (Brown et al., 1970; Collier & Katz, 1970; Nishi, 1970; Ginsborg, 1971). Hence, the increase in threshold dose found in the study of Volle and Koelle (1961) may well be an elevation of the threshold for activation of nicotinic receptors.

Brown (1969) used both nictitating membrane contraction and surface recording of ganglionic depolarization and reported that denervation increases the sensitivity of the sympathetic ganglion to ACh but not to carbachol. He concluded that the supersensitivity to ACh is probably due to a marked reduction in ganglionic AChE following presynaptic denervation (cf. Sawyer & Hollinshead, 1945; Koelle, 1955). However, he did not distinguish whether the increased sensitivity to ACh or the decreased sensitivity to carbachol is due to a change in nicotinic or muscarinic response. Bokri et al. (1963) showed that the response of denervated cat superior cervical ganglia can be enhanced to the same degree as in normal ganglia by pretreating with an anti-ChE agent, tetraethylpyrophosphate (TEPP). They concluded that a decrease in ganglionic AChE should not be responsible for the increase in the ganglionic sensitivity to ACh. The present

data support their conclusion in that eserine enhanced the ACh muscarinic activity in both control and denervated ganglia. In addition, the carbachol muscarinic response was also increased; this further demonstrates that the increased sensitivity to ACh was not solely due to a reduction in ganglionic AChE. Furthermore, recent histochemical studies have shown that ganglionic AChE is distributed in both pre- and postganglionic elements; preganglionic denervation does not result in a total disappearance of ganglionic AChE (Dhar, 1958; Klingman & Klingman, 1969).

A decrease in nicotinic response to an intra-arterial injection of ACh on cat superior cervical ganglia has been reported by Green (1969) and Vickerson and Varma (1969) after making appropriate adjustment for the increased sensitivity of the nictitating membrane to norepinephrine after denervation. Thus, the present results which show that denervation depressed the ganglionic nicotinic response are well in accord with other previous studies.

#### Enhancement of Muscarinic Response after Denervation

The present study shows that denervation rendered the rabbit superior cervical ganglion more sensitive to muscarinic depolarizing agents. The dose-response relationships obtained with sucrose-gap method and intracellular techniques indicate that the minimum effective concentration in eliciting a detectable muscarinic depolarization was 10 to 50 fold lower, and the maximal depolarization was 60 to 80 per cent



higher in denervated ganglia. The enhanced muscarinic depolarization was fully antagonized by a low concentration of atropine ( $1 \times 10^{-5} M$ ).

In contrast to the present result, Green (1969) observed a decrease in the muscarinic response of denervated cat superior cervical ganglia. He measured the ganglionic response indirectly by recording nictitating membrane contraction following drug application. This method, however, depends upon so many parameters that it is often difficult to evaluate the sensitivity of the ganglion simply from the amplitude of the nictitating membrane contraction. The difficulty may arise from the following possible factors: (1) the frequency of postganglionic discharges is dependent upon the time course of ganglionic depolarization induced by drug application; (2) the effect of applied drug depends upon the condition of local vascular circulation, and (3) contraction of the nictitating membrane is dependent upon the amount of norepinephrine being liberated from the postganglionic trunks, the sensitivity of the nictitating membrane to norepinephrine and the contraction force of the smooth muscle contractile system. Unless these factors are experimentally controlled or remain unchanged after denervation, the validity of employing nictitating membrane contraction as an indicator for ganglionic sensitivity is questionable. Indeed, several studies have shown that the sensitivity of the nictitating membrane to catecholamines following decentralization (sectioning of the preganglionic

nerve trunk) or denervation (sectioning of the postganglionic nerve trunk) is increased (Innes & Kosterlitz, 1954; Chein, 1960; Bokri et al., 1963; Trendelenburg, 1963; Langer & Trendelenburg, 1966; Langer et al., 1967; Green, 1969; Vickerson & Varma, 1969). Although Green (1969) has corrected the nictitating membrane supersensitivity to norepinephrine after denervation, the remaining uncontrolled factors enumerated above may contribute to the ambiguity of his observation.

The experiment conducted by Ambache et al. (1956) first indicated that the contraction of nictitating membrane can be elicited by a smaller dose of muscarine injected to the denervated ganglion. Jones (1963) confirmed their finding and reported that denervation indeed sensitizes the sympathetic ganglion to muscarinic agents.

Takehige and Volle (1963) used the postganglionic discharge method and showed that the amount of ACh required to activate the nicotinic component is the same for both normal and denervated ganglia. On the other hand, the threshold dose for activation of the muscarinic component is substantially lower in denervated than in normal ganglia. They suggested that denervation results in an 'unmasking' of muscarinic receptors.

Bokri et al. (1963) reported that the denervation supersensitivity of cat superior cervical ganglia to ACh is depressed by atropine; on the contrary, hexamethonium failed to antagonize the denervation supersensitivity produced by

ACh (Perry & Reinert, 1954; Bokri et al., 1963). This indicates that nicotinic receptors are not involved in the production of supersensitivity which is consistent with the present idea that muscarinic receptors are more sensitive to ACh after denervation. The possibility that denervation supersensitivity may be a result of an increase in the participation of muscarinic receptors and/or the area occupied by these receptors has been proposed by Bokri et al. (1963). A similar conclusion was reached by Vickerson and Varma (1969) who suggested that denervation causes an increase in the number and/or sensitivity of muscarinic receptors.

The results obtained directly from the whole ganglion as well as the single ganglion cell agree well with previous findings that the denervated sympathetic ganglion becomes supersensitive to muscarinic agents. In addition, the present results show that even though the muscarinic response was enhanced after denervation, its amplitude was smaller than that of a control nicotinic response. This indicates that the postganglionic discharges induced by ACh or carbachol would be less in denervated than in control ganglia, which is compatible with the finding of Volle and Koelle (1961) in which they showed diminished postganglionic discharges induced by carbachol after denervation.

#### Sensitivity of the Sympathetic Ganglion to Dopamine

Both sucrose-gap experiments and intracellular studies show that the hyperpolarization of ganglion cells induced by

bath perfusion with dopamine was slightly augmented by denervation. However, in the study of iontophoretic application of ACh, the hyperpolarization which followed the fast ACh potential was not observed in control ganglion cells, whereas it was often observed in denervated ganglion cells. This hyperpolarization was found to resemble the ganglionic slow i.p.s.p. proposed by Libet (1970).

Since the hyperpolarizing response resulting from bath perfusion with dopamine was only slightly increased after denervation, the markedly enhanced hyperpolarization induced by iontophoretic application of ACh to the denervated ganglion cells cannot be explained by an increased sensitivity of the ganglionic postsynaptic membrane to dopamine. The increase in hyperpolarizing response induced by iontophoretic application of ACh should be, then, due to the increased liberation of dopamine from chromaffin cells. In fact, chromaffin cells are mainly endowed with muscarinic receptors (Libet, 1970), which according to the present study are sensitized to ACh after denervation. This may explain the present observation that the hyperpolarization induced by bath perfusion with dopamine was not increased to any significant degree after denervation, but the injected ACh stimulated the sensitized muscarinic receptors on the chromaffin cells which released a larger quantity of endogenous dopamine. As mentioned previously, the sensitivity of the nictitating membrane to norepinephrine is increased after decentralization or denervation; however, the degree of

supersensitivity produced by decentralization is much less than following denervation (Langer et al., 1967). If the dopamine release is mediated di-synaptically as proposed by Libet (1970), the increase in postsynaptic hyperpolarizing response to dopamine is analogous to the decentralized nictitating membrane; the resultant increase in dopamine hyperpolarization should be relatively small.

It should be emphasized from the present study that the main site of action of dopamine on sympathetic ganglia is presynaptic; it leads to a reduction of ACh output from the presynaptic nerve terminals. The postsynaptic action of dopamine, i.e., a weak hyperpolarization, seems to play a secondary role, if any, in the blockade of ganglionic transmission. Although the after-discharge of sympathetic ganglion cells, which is triggered by the slow e.p.s.p. (Nishi & Koketsu, 1966, 1968 a & b), is suppressed during the production of the i.p.s.p. (Koketsu & Nishi, 1967), the amplitude of slow i.p.s.p. is generally too small to inhibit the impulse generation by the fast e.p.s.p.

#### Passive Membrane Properties

One of the factors which determines the amplitude of a synaptic potential is input resistance (effective resistance,  $R_e$ ) (Katz & Thesleff, 1957). The present study shows that effective resistance was not changed after denervation; this implies that the reduction of the nicotinic response or the augmentation of muscarinic response is not the result of a

change in effective membrane resistance.

The effective resistance is determined by the membrane resistance for a unit area ( $R_m$ ) and the cell surface area ( $S$ ); namely,  $R_e = R_m/S$ . It is possible that the unaltered  $R_e$  after denervation may be due to either a simultaneous decrease or increase of both  $R_m$  and  $S$ . Since  $R_m$  cannot be determined directly, the cell diameter was measured in this study to provide indirect information of  $R_m$  after denervation.

The results show that the cell diameter was not changed appreciably after denervation. Similar findings were obtained in denervated frog (Hunt & Nelson, 1965) and guinea pig (McLachlan, 1974) sympathetic ganglia. According to the equation, if two of the parameters,  $R_e$  and  $S$ , remained the same after denervation  $R_m$  should remain unchanged. The unaltered  $R_m$  implies that the potassium permeability ( $P_K$ ) at rest is not affected by denervation, since  $R_m$  at rest is mainly dependent upon  $P_K$  as suggested by Woodward et al. (1969). They estimated the relative resting membrane permeabilities of K ( $P_K$ ), Na ( $P_{Na}$ ) and Cl ( $P_{Cl}$ ) in the rabbit superior cervical ganglion and found  $P_K : P_{Na} : P_{Cl}$  to be 1 : 0.06 : 0.02. The  $P_K$  at rest is much larger than  $P_{Na}$  and  $P_{Cl}$ , suggesting that the resting membrane potential is largely determined by  $P_K$  (Woodward et al., 1969). This is again consistent with the present observation that the resting membrane potential was not changed after denervation.

The unaltered resting membrane potential implies that the ionic content of ganglion cells is not altered after denervation. It is generally agreed that the intracellular ionic content of a nerve cell is maintained relatively constant by a Na-K pump system (cf. Hodgkin & Keynes, 1955). It is reasonable to assume, therefore, that the activity of the Na-K pump is well preserved after denervation.

Hence, the reduction of the Na pump dependent hyperpolarization (Brown et al., 1972; Lees and Wallis, 1974) following the removal of ACh was not the result of a diminished activity of Na-K pump but was due to actual diminution of the nicotinic response after denervation.

The membrane time constant of cervical sympathetic neurons was also found to be unchanged after denervation, indicating that the membrane capacitance ( $C_m$ ) was unaffected by denervation, since the time constant is the product of  $C_m$  and  $R_e$ . It is known that  $C_m$  is related to the dielectric constant ( $\epsilon$ ) and thickness ( $d$ ) of the membrane, as expressed by the following equation (cf. Hoyle, 1957):

$$C_m = 8.8 \times 10^{-6} \epsilon/d.$$

The structure and dimension of the membrane proteolipid should be well preserved following denervation.

Hunt and Nelson (1965) observed no change in membrane potential and capacitance but an increased membrane resistance and time constant in denervated frog sympathetic neurons. However, they did not specify the possible reason

for this observed alteration in membrane resistance and time constant. Since the membrane resistance is related to the ionic permeability of the membrane, the increased resistance implies a decrease in membrane permeability. As the resting membrane potential was unchanged in the experiments of Hunt and Nelson (1965), denervation probably caused a nonselective decrease of  $P_{K}$ ,  $P_{Na}$  and  $P_{Cl}$  in frog sympathetic neurons; in other words, the ratio of  $P_{K} : P_{Na} : P_{Cl}$  must have remained constant. Similar to the present observation, McLachlan (1974) reported no change in the passive membrane properties of denervated guinea pig sympathetic neurons; i.e., resting membrane potential, input resistance, membrane time constant and cell capacitance remained the same after denervation.

#### Active Membrane Properties

The measurement of threshold for ganglion cell excitation is particularly important with regard to the observation of Volle and Koelle (1961) that the carbachol induced postganglionic discharges are diminished after denervation and the finding of Green (1969) that ACh injected intra-arterially is less effective in eliciting nictitating membrane contraction. The lowered responses observed in their experiments could be due to either the elevation of electrical threshold or lowering of chemo-sensitivity. The possibility of alteration of electrical threshold was explored in



this study by measuring various parameters of action potentials induced by direct intracellular stimulation. No significant change was observed in any of the parameters of action potentials measured after denervation. The present data are supported by similar observations of Hunt and Nelson (1965) and McLachlan (1974) who reported no change in the action potential parameters of frog and guinea pig sympathetic neurons after denervation. Thus, the hyposensitivity of denervated ganglion cells to cholinergic agents as proposed by Volle and Koelle (1961) and Green (1969) is not likely to be due to the elevation of membrane electrical threshold, but is due to a reduction in the ganglionic response that triggers the postganglionic discharges. It would seem that whatever mechanism underlies the genesis of the hyposensitivity or hypersensitivity of cholinergic receptors after denervation, this mechanism is independent of the mechanism regulating the membrane electrical properties.

#### Equilibrium Potential of the Nicotinic Subsynaptic Membrane

The  $E_{\text{epsp}}$  indicates the driving force for the movement of ions involved in the electrogenesis of nicotinic subsynaptic membrane depolarization. A change in  $E_{\text{epsp}}$  has a diagnostic value for the determination of the ionic permeabilities involved in synaptic potentials. For example, a shift of  $E_{\text{epsp}}$  to a hyperpolarizing direction after denervation would cause a lowering in the driving force of nicotinic

depolarization resulting in a diminished nicotinic depolarization induced by cholinergic agonists. A shift of  $E_{\text{epsp}}$  to a depolarizing direction would give the opposite effect.

The data obtained in this study show that there is a slight increase in the mean  $E_{\text{epsp}}$  of denervated ganglion cells; however, the difference is statistically insignificant. It is highly improbable that this slight increase would be responsible for nearly 50 per cent reduction in nicotinic depolarization. The present data well agree with the findings of Axelsson and Thesleff (1959) and Magazanik and Potapova (1969) as they observed no difference in the reversal potentials of ACh induced by iontophoretic application of ACh to control and denervated cat tenuissimus muscle and rat diaphragm muscle.

Calculation of  $\Delta g_{\text{Na}}/\Delta g_{\text{K}}$  was based on the assumption that the equilibrium potential for Na ( $E_{\text{Na}}$ ) and K ( $E_{\text{K}}$ ) remained unchanged after denervation. This assumption seems to be relevant in that the resting membrane potential, the action potential amplitude and the reversal potential of the after-hyperpolarization were not changed after denervation, indicating  $E_{\text{Na}}$  and  $E_{\text{K}}$  remained constant following denervation. The calculated values of  $\Delta g_{\text{Na}}/\Delta g_{\text{K}}$  of control and denervated ganglion cells show essentially no change following denervation, suggesting that the reduced nicotinic response was due to the reduction of the total conductance change in the nicotinic subsynaptic membrane ( $\Delta g_{\text{Na}} + \Delta g_{\text{K}}$ ), while the ratio

of Na conductance change to K conductance change ( $\Delta g_{Na}/\Delta g_K$ ) was kept constant. The situation is quite similar to the action of d-Tc on the endplate membrane, in which the total conductance is decreased while the ratio is not affected (Takeuchi & Takeuchi, 1960). Such a change in the total conductance after denervation raises two alternative possibilities in regard to the nicotinic receptors; namely, the denervation decreases the density of nicotinic receptors, or the unitary effect of ACh, that is the Na and K conductance change induced by activation of a unitary receptor is decreased.

#### Drug-Receptor Interaction

ACh is thought to combine with specific macromolecules, the receptors, which control the channels in the postsynaptic membrane for the passage of certain ions. Drug-receptor interaction is the first of several sequential steps in the initiation of the postsynaptic membrane potential. In a simple mathematical interpretation, the 'affinity' of a drug to the receptor is proportional to the reciprocal of  $K_a$ , the dissociation constant of the drug-receptor complex (Ariëns et al., 1957). Possession of an 'affinity' to the receptor is not sufficient to induce a drug effect; a drug must interact 'effectively' with the receptors. The contribution of a single drug-receptor complex to a unit of drug effect is called the 'intrinsic activity' (Ariëns et al., 1957).

Mathematically the 'intrinsic activity' of a drug is inversely proportional to the number of receptors that have to be occupied to induce a certain effect.

It is possible that denervation somehow alters the physico-chemical properties of receptors of the sympathetic neurons in such a way that the 'affinity' and/or the 'intrinsic activity' of a drug to the receptor is either increased or decreased. Hence, the alteration in nicotinic and muscarinic response may be a result of a change of 'affinity' and/or 'intrinsic activity' of a drug to the receptor.

The results in this study show that  $K_a$  for ACh-nicotinic receptor complex obtained from double reciprocal plots is not appreciably changed after denervation; the  $K_a$  for ACh-muscarinic receptor complex is also not significantly altered in denervated preparation. The estimation of the number of ACh molecules that attach to a single nicotinic or muscarinic receptor (cf. Takeuchi & Takeuchi, 1969) revealed that the drug-receptor molecule ratio remains the same (1:1) after denervation. The results, therefore, suggest that neither the drug 'affinity' nor the ratio of drug-receptor complex is affected after denervation.

#### Neurotrophic Influences of Nerve on Receptors

Miledi (1960 a & c, 1962) hypothesized that the increased sensitivity of extra-junctional area of denervated

muscle fiber to ACh is a result of spreading of ACh receptors from the junctional region to the non-junctional area. He theorized that the motor nerve exerts a restraining action on the sensitivity of ACh receptors; a removal of this restraining factor, such as denervation, allows the receptors to proliferate along the entire muscle fiber surface. He further speculated that there is a 'receptor controlling factor' being liberated from the motor nerve which ultimately plays the role of regulating the chemo-sensitive area. He did not elaborate on the chemical property of this unidentified controlling factor; however, he did exclude the possibility of ACh being the neurotrophic factor (Miledi, 1960 c).

Thesleff (1960) proposed that ACh may be the neurotrophic factor, since in a botulinum toxin treated nerve-muscle preparation, ACh sensitivity of the non-endplate region is markedly increased, which resembles denervation supersensitivity. The mode of action of botulinum toxin has been suggested to impair the release of ACh from cholinergic nerve terminals (Thesleff, 1960). Therefore, it is assumed that in denervated muscle as in botulinum toxin treated muscle lack of transmitter output is causally related to the receptor chemo-sensitivity. It is conceivable that the release of some other unidentified substance or substances may also be impaired by botulinum toxin or denervation. Therefore it is uncertain whether the trophic

factor is a unique neurohumoral substance or ACh (cf. Guth, 1968).

It is generally accepted that there are physiological and biochemical changes that occur when muscle is denervated (cf. Guth, 1968). There is a distinct possibility that the alterations in chemo-sensitivity observed in denervated sympathetic ganglia may be due to a lack of trophic factor liberated from presynaptic nerve terminals. Since the modes of generation of nicotinic and muscarinic potentials are entirely different, it is conceivable that presynaptic nerve may exert different trophic influences on nicotinic and muscarinic receptors; that is, there may be a factor which promotes the proliferation of nicotinic receptors and there may be a factor which restrains the activity of muscarinic receptors under normal conditions. The removal of these trophic factors, such as by denervation, may result in a reduction in nicotinic receptors and a spreading of muscarinic receptors. Though much indirect evidence has been suggested by Miledi (1962) that the motor nerve controls the induction of receptors on muscle membrane, no direct evidence has yet been secured to substantiate this theory. One of the most crucial pieces of evidence that would unequivocally confirm or refute the neurotrophic influences on receptor induction is the characterization and identification of this substance or substances; this evidence is not yet available. Until the identity of this neurotrophic substance or sub-

stances is established, the theory of neurotrophic induction of receptors remains speculative.

#### Redistribution of Nicotinic and Muscarinic Receptors

It is well demonstrated that there is an increase in the chemo-sensitive area in denervated mammalian (Axelsson & Thesleff, 1959) and amphibian (Miledi, 1960 a & b) muscle fiber. Recently this observation has been extended to neurons. Kuffler et al. (1970) have shown that after denervation the extra-synaptic portion of the frog cardiac parasympathetic ganglion cell membrane becomes highly sensitive to focally applied ACh; the extra-synaptic area of the normal cell membrane is relatively unresponsive to focally applied ACh. However, they did not explore further the pharmacological property of the extra-junctional receptors. It is conceivable that the pharmacological response of denervated parasympathetic ganglia may well resemble that of denervated sympathetic ganglia observed in this study.

Binding of labelled ligands to receptor molecules has been extensively employed for the last decade to characterize and quantify receptor molecules (Miledi & Potter, 1971; De Robertis et al., 1972). De Robertis et al. (1972) showed that in rat diaphragm there is an increase in the binding of  $^{14}\text{C}$ -ACh after denervation, especially in the non-endplate region. The chief peril of inferring receptor number from the number of molecules bound is nonspecific

binding. In the present study, the nicotinic response is depressed and muscarinic response is enhanced after denervation. On the assumption that sensitivity is simply an index of the local density of receptor units, and the unit receptor reactivity is the same after denervation, it is likely that a decreased nicotinic response may be due to a reduction in the number of nicotinic receptors and an enhanced muscarinic response may well be an increase in the density of muscarinic receptors. The contention is supported by the observation of Miledi (1960 a) in which he concluded that in denervated frog sartorius muscle there is an increase in the chemo-sensitive area with no actual increase in the sensitivity of the individual receptor. In addition, others have suggested that the enhanced muscarinic activity in denervated cat superior cervical ganglion may be a result of an increase in the number of muscarinic receptors (Bokri et al., 1963; Vickerson & Varma, 1969).

There is a possibility, among many others, that the mutual spatial arrangement of neighboring nicotinic and muscarinic receptors may be intimately related to the operation of ionic channels. Hence, a reduction in the number of nicotinic receptors may somehow facilitate the muscarinic receptor-agonist interaction; conversely, an increase in muscarinic receptors may somehow hinder the nicotinic receptor-agonist interaction.



It is of interest to note that the higher the species phylogenetically the more dominant are the nicotinic receptors on the muscle fiber (Magazanik et al., 1963). The curare/atropine coefficient which has been used to characterize quantitatively the nicotinic and muscarinic receptors has been shown to be smaller in lower animals, and this ratio decreased after denervation (Magazanik et al., 1963). A similar finding has been reported in rat diaphragm, namely that denervation modifies the muscle sensitivity to curare and atropine; curare is less effective and atropine is more effective in blocking the ACh potential induced by focal application of ACh (Beranek & Vyskocil, 1967). Jenkinson (1960) made a similar observation on frog sartorius muscle. It is tempting to speculate in the framework of these findings that an organ undergoes changes after denervation which cause its properties to revert to those of earlier evolutionary stage. In case of the sympathetic ganglion, the nicotinic receptor may lose its specific properties and transform to the more primitive muscarinic receptors. This may explain why there is a simultaneous decrease in nicotinic response and an increase in muscarinic response.

### Conclusions

The effect of chronic preganglionic denervation on the rabbit superior cervical ganglion was investigated with

the sucrose-gap method and intracellular microelectrode techniques. Analysis of the results obtained from this study gives the following conclusions:

1) the nicotinic sensitivity of the rabbit superior cervical ganglion to nicotinic agonists is substantially decreased after chronic denervation;

2) the muscarinic sensitivity of the denervated superior cervical ganglion to muscarinic agonists is markedly increased;

3) the enhanced muscarinic response of the denervated ganglion can be completely blocked by atropine;

4) the hyperpolarizing response of the denervated ganglion to dopamine is slightly enhanced;

5) the passive cell membrane properties are not altered significantly after chronic denervation;

6) the active membrane properties are not affected appreciably by denervation;

7) the equilibrium potential of the nicotinic subsynaptic membrane appears to be the same before and after denervation;

8) the total conductance change ( $\Delta g_{Na} + \Delta g_K$ ) of the activated nicotinic subsynaptic membrane is decreased while the ratio of  $\Delta g_{Na}/\Delta g_K$  of the activated nicotinic subsynaptic membrane is not changed appreciably after denervation;

9) the cell diameter is within normal range after denervation;

10) the drug-receptor affinity remains constant after denervation.

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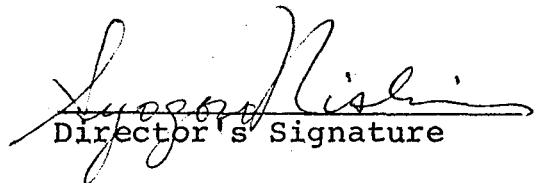
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The final copies have been examined by the director of the dissertation and the signature which appears below verifies the fact that any necessary changes have been incorporated and that the dissertation is now given final approval by the Committee with reference to content and form.

The dissertation is therefore accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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