DIGITALIS TOXICITY: PRIMARY SITES OF DRUG ACTION ON THE SYMPATHETIC NERVOUS SYSTEM

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Lynne Christine Weaver

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Major professor

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ABSTRACT

DIGITALIS TOXICITY: PRIMARY SITES OF DRUG ACTION ON THE SYMPATHETIC NERVOUS SYSTEM

By

Lynne Christine Weaver

Cardiac glycosides induce both increases and decreases in the activity of peripheral sympathetic nerves. These neural effects have been proposed to contribute to the induction of cardiac arrhythmias caused by toxic doses of digitalis. Drug actions on various sites such as the central nervous system, ganglia, chemoreceptor and baroreceptor afferent nerve fibers and peripheral efferent nerve fibers can affect sympathetic activity. Digitalis also increases phrenic nerve activity and causes hyperventilation. These effects also could be produced centrally or peripherally. The relative contribution of the various possible sites of drug action to these neural effects of digitalis has not been well defined.

Four groups of experiments were conducted to identify the primary sites involved in the sympathetic neural effects of digitalis. First, since direct central effects of digitalis on sympathetic discharge had not been documented, the central action of ouabain on sympathetic outflow was examined on peripheral

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sympathetic nerves in baroreceptor and chemoreceptor denervated cats. Ouabain was injected into 128 vasoconstrictor or cardioaccelerator sites in the medulla or hypothalamus. Electrical stimulation of 24% of these sites evoked arrhythmias and stimulation consistently caused marked increases in heart rate, blood pressure and peripheral nerve activity. But ouabain had several effects, inducing either no change or increases or decreases in spontaneous activity of vasoconstrictor and cardioaccelerator nerves. Effects of ouabain were also observed on signal-averaged potentials evoked in these nerves by electrical stimulation of the medullary or hypothalamic sites of injection. The amplitude of the evoked potentials was either increased, decreased or left unchanged. In general, spontaneous and evoked activity were inhibited by ouabain more frequently than they were enhanced. The pattern of nervous responses to ouabain did not relate to the dose of drug or to the anatomical site of injection. Medullary and hypothalamic injections of ouabain often produced large changes in blood pressure, heart rate, and nerve activity, but these effects were not accompanied by alterations in cardiac rhythm. Thus, central microinjections of ouabain produced heterogeneous patterns of effects on activity of peripheral sympathetic nerves, and these microinjections were not sufficient to evoke cardiac arrhythmias in cats with sectioned cranial nerves IX and X.

The goal of the next group of experiments was to define the relative contribution of peripheral and central sites of drug action following intravenously administered digitalis. Digoxin was

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administered intravenously to cats to study its effects on activity of preganglionic splanchnic or postganglionic inferior cardiac nerves in the presence or absence of chemoreceptor and baroreceptor reflexes. In cats with intact reflexes, arrhythmic doses of digoxin had diverse effects on postganglionic activity. In some cats digoxin increased activity and in others it decreased activity. In contrast, digoxin consistently caused large progressive increases in postganglionic activity when baroreceptors and chemoreceptors had been denervated. Digoxin inhibited preganglionic nerve activity in cats with intact reflexes and had no effect in those without chemoreceptor and baroreceptor reflexes. Since digoxin only inhibited preganglionic or postganglionic nerve activity in the presence of intact baroreceptor afferents, these are the apparent site of digoxin-induced inhibition. Increases in activity above control in response to digoxin were observed only in postganglionic nerves. This suggested that digoxin acts on the ganglion to increase sympathetic activity. Since digoxin had no discernible effect on preganglionic activity when baroreceptor and chemoreceptor afferent input had been eliminated, these data were not consistent with the hypothesis that a primary site of drug action is in the central nervous system.

To further test this hypothesis, effects of digoxin were observed on a nerve which is not of sympathetic origin, the phrenic nerve. This nerve was also selected because digitalis enhances its activity. In cats with intact chemoreceptor and baroreceptor afferent nerves, digoxin caused marked increases in phrenic nerve

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activity, whereas in the absence of afferent influences digoxin had no effect. This suggested that effects of digoxin on respiration were primarily dependent upon afferent input to respiratory neurons. Possibly digoxin had a subliminal effect on central neurons, which increased phrenic activity only in the presence of excitatory afferent input. Probably effects of digoxin on respiration were due to drug actions on peripheral sites on afferent nerves having excitatory influence on respiration.

Although the central injection experiments showed that digitalis can have prominent effects on sympathetic nerve activity, the second two groups of experiments raised questions concerning whether central actions of these drugs were responsible for altered sympathetic nerve activity following intravenously administered digitalis. The last group of experiments was designed to reveal any biochemical effect of digitalis in the brain which might suggest a central action of the drug. The concentrations of digoxin in the cerebrospinal fluid were determined following doses of digoxin which have been shown to evoke neural effects. Tritiated digoxin (20 μg/kg) was injected intravenously into cats every 15 min until ventricular fibrillation occurred. Cerebrospinal fluid and serum concentrations were determined. Nanomolar drug concentrations were present in cerebrospinal fluid. These concentrations were approximately 10% of total serum digoxin concentrations but only slightly lower than unbound serum digoxin concentrations.

Since inhibition of Na^+-K^+-ATPase is often associated with pharmacological effects of digitalis, effects of nanomolar

concentrations of digoxin on Na^+-K^+-ATPase activity from 8 brain areas of the cat were determined *in vitro*. The maximum concentration of digoxin found in CSF (2×10^{-8} m) inhibited Na^+-K^+-ATPase only slightly (10-20%). Activity of Na^+-K^+-ATPase from the brains of cats which had been treated with lethal doses of digitoxin was also examined. After ventricular fibrillation, the cat brains were removed and Na^+-K^+-ATPase activity and ouabain binding to Na^+-K^+-ATPase were determined in 8 areas. No inhibition of ATPase or decreased ouabain binding was observed in any area. Thus, it appeared that relatively acute treatment with toxic doses of digitalis in the cat did not cause significant inhibition of brain Na^+-K^+-ATPase activity.

The data obtained in these studies showed that digitalis does have prominent effects on sympathetic nerve activity. The data strongly suggest that these effects stem from drug actions in the ganglion and on baroreceptor afferent nerves. Digitalis also has prominent effects on phrenic nerve activity which are dependent upon intact afferent influence on central respiratory neurons. No electrophysiological or biochemical evidence for prominent drug actions in the brain was obtained. Therefore, it is concluded that altered sympathetic nerve activity produced by digitalis results primarily from drug actions in the peripheral nervous system.

DIGITALIS TOXICITY: PRIMARY SITES OF DRUG ACTION ON THE SYMPATHETIC NERVOUS SYSTEM

By

Lynne Christine Weaver

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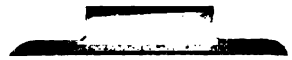

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INTRODUCTION

General Background

The drugs commonly referred to as cardiac glycosides are derived from plants and can be obtained from digitalis, strophanthus and squill. The entire group of drugs is often simply referred to as digitalis. Digitalis has been used medicinally for centuries but the first extensive description of the medical uses of digitalis was that of William Withering in 1785 in his famous book entitled *An Account of the Foxglove and Some of Its Medical Uses: With Practical Remarks on Dropsy and Other Diseases*. Withering used digitalis particularly for its diuretic action in the treatment of dropsy but also noted its powerful action on the heart. Thus, digitalis has been used for various ailments, including those of the heart, since the time of William Withering. It has been primarily used for the treatment of congestive heart failure only in the past 50 years. Cattell and Gold (1938) showed that ouabain enhances the contractile force of isolated "failed" cat papillary muscle. It has since been assumed that the pharmacological effect of digitalis in the treatment of heart failure is an action upon the mechanical properties of the heart to improve inotropy. However, when cardiotoxicity is produced by high doses of digitalis, the electrical properties of the heart are affected resulting in

arrhythmias. This effect is often clinically associated with symptoms of neural origin. Actions of cardiac glycosides on the nervous system have been implicated since the time of William Withering. Clinically well known central effects of digitalis include disturbances of color vision, blurred vision, headache and irritability (Batterman and Gutner, 1948). The most dramatic neural effect of digitalis is its ability to induce seizures such as those reported by Gold *et al.* (1947) in rats following intravenous administration of red squill. Nausea and vomiting, another side effect of digitalis, have been interpreted by Borison and Wang (1951) and Gaitonde *et al.* (1965) as the result of drug action on the medullary chemoreceptor trigger zone. The nausea and vomiting caused by digitalis originally were attributed to gastric irritation which is partly due to saponin contained in the leaves of the digitalis plant. Later it was demonstrated that intravenous and intra-arterial injections of cardiac glycosides can cause nausea. When Borison and Wang (1951) and Gaitonde *et al.* (1965) eliminated the emetic responses to cardiac glycosides by ablating the medullary chemoreceptor trigger zone, they concluded that the emetic responses were due also to central actions of the drug.

Sympathetic Neural Effects of Digitalis

Recently, attention has been drawn to neural effects of digitalis on cardiovascular function. Digitalis-induced alterations in sympathetic activity have been proposed to contribute to the induction of cardiotoxic arrhythmias (Cairoli *et al.*, 1961). This is feasible since alterations in sympathetic activity to the heart can

induce cardiac arrhythmias in the absence of any drug. Beatie *et al.* (1930) first showed that extrasystoles could be elicited by stimulating the wall of the third ventricle in the cat. Fuster and Weinberg (1960) and Hockman *et al.* (1966) induced arrhythmias as severe as ventricular fibrillation by electrically stimulating the diencephalic and mesencephalic reticular formation of cats and dogs. Manning and Cotten (1962) also evoked cardiac arrhythmias from the hypothalamus and attributed the rhythm disorders to simultaneous activation of sympathetic and parasympathetic innervation of the heart. Tachyarrhythmias have been evoked by stimulation of individual peripheral cardiac nerves (Armour *et al.*, 1972).

Digitalis alters neural influences on the heart producing both increases and decreases in the activity of cardiac sympathetic nerves. Gillis (1969) first reported biphasic effects of ouabain on preganglionic cardiac sympathetic nerves. Inhibition of activity at lower doses was followed by enhancement at higher doses which coincided approximately with the onset of cardiac arrhythmias. He suggested that this enhanced activity contributed to the induction of arrhythmias. McLain (1969) reported both increases and decreases in activity of postganglionic inferior cardiac nerves occurring in response to cardiac glycosides. He observed consistently increased sympathetic activity at the highest doses of digitalis. Roberts (1970) showed that subarrhythmic doses of ouabain diversely altered the excitability of preganglionic and postganglionic cardiac nerves to electrical stimulation. The chronotropic response to cardiac nerve stimulation became labile and variable after ouabain

administration. In a later report Roberts *et al.* (1974) described diverse responses to arrhythmic doses of ouabain occurring in different filaments of the same postganglionic cardiac nerve. They suggested that ouabain had non-uniform effects on cardiac adrenergic nerve fibers, causing increased discharge in some fibers and decreased discharge in others. They proposed that this action of ouabain on sympathetic nerves could cause non-uniform changes in excitability and conduction in the heart resulting in arrhythmias.

The early inhibition of sympathetic activity may be of therapeutic value in slowing the rapid heart rates of congestive heart failure. The late changes (whether increases or diverse kinds of alterations in activity) appear to coincide with the onset of cardiac arrhythmias. Effects of digitalis on sympathetic nerves may contribute to the genesis of arrhythmia. Alternatively, basic sympathetic tone but not digitalis induced alterations in activity may be important in supporting or magnifying direct cardiac actions of digitalis. Roberts *et al.* (1963) showed that pretreatment of cats with reserpine or βTM10 (a drug which prevents the release of catecholamines) more than doubled the dose of acetyl strophanthidin needed to induce arrhythmia. After doses of reserpine, the incidence of extra beats induced by ouabain in isolated cat papillary muscle was also reduced. Erlij and Mendez (1964) reduced adrenergic influences on the hearts of dogs and cats by treating them with reserpine or by removing thoracic sympathetic chains and adrenal glands. They found that this procedure increased the lethal dose of digitoxin or ouabain by at least 30%. Levitt *et al.* (1973)

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compared the dose of ouabain needed to induce arrhythmia and fibrillation in intact cats with the dose needed in cats with transected spinal cords. They found that the arrhythmic and lethal doses in spinal cats were twice that in intact cats.

Decreased sympathetic activity could reduce the toxicity of cardiac glycosides related to associated decreased blood pressure, heart rate and body temperature. Raines *et al.* (1967) showed that cats with heart rates greater than 200 beats per min developed arrhythmias at lower doses of ouabain than did cats with heart rates less than 200 beats per min. They considered that tachycardia was a significant factor in the genesis of arrhythmia. However, Ciofalo *et al.* (1967) showed that changes in blood pressure, heart rate and body temperature alone did not modify the capacity of ouabain to induce ventricular arrhythmias in cats. Thus, surgical or pharmacological reductions in sympathetic activity diminish the capacity of digitalis to cause cardiac arrhythmias. This strongly suggests that sympathetic influences on the heart contribute to the arrhythmogenic effects of digitalis.

Changes in the activity of postganglionic nerves entering the heart could stem from drug actions on many sites within the neuraxis. Digitalis enhances carotid sinus reflexes (Heymans, 1932; Abiko, 1965) and increases traffic in aortic and carotid sinus nerves (McLain, 1970), thus evoking alterations in central sympathetic outflow. Schmitt (1958a,b) showed that digitalis increases chemoreceptor discharges in the carotid sinus nerve and in the carotid body. Baroreceptor activity in the carotid sinus nerve is also

enhanced by digitalis, an effect which is not dependent upon increases in blood pressure (Quest and Gillis, 1971, 1974).

Digitalis has also been proposed to act in the central nervous system to increase sympathetic outflow. Weinberg and Haley (1955) injected strophanthin-K into the third ventricle of anesthetized dogs and evoked cardiovascular responses and arrhythmias. These effects could be blocked by intravenous administration of hexamethonium. Bircher (1963) evoked cardiac arrhythmias in dogs by injecting deslanoside into the fourth ventricle. These arrhythmias were partially reversed by spinal cord transection. Basu Ray *et al.* (1972) injected ouabain into the hypothalamus or cerebral ventricles of cats, producing cardiac arrhythmias and changes in blood pressure and respiration. While these central injection experiments showed that central effects of digitalis can cause cardiovascular responses, they do not prove that neural effects of intravenously administered drug stem from central actions. However, Gillis (1972) argued that since digitalis simultaneously affects sympathetic, vagus and phrenic nerves, the drug must act centrally to produce responses in all three nerves at once.

A prominent site for drug action in the peripheral efferent sympathetic nervous system is the ganglion. Konzett and Rothlin (1952) perfused the intact or decentralized superior cervical ganglion of the cat with various cardiac glycosides. They observed responses of the nictitating membrane to chemical or electrical stimulation and reported that small doses of cardiac glycosides potentiated the responses to such stimuli. At higher doses of

digitalis they reported that the potentiating effect could be followed by a paralyzing effect. Their results were confirmed by Perry and Reinert (1954). Acetylcholine release from the superior cervical ganglion is also enhanced by digitalis (Birks, 1963).

Finally, digitalis can increase the excitability of peripheral autonomic nerves. Ten Eick and Hoffman (1969) stimulated decentralized preganglionic and postganglionic sympathetic nerve trunks and recorded action potentials from pre- and postganglionic nerves. Ouabain decreased the frequency of stimulation required to evoke a maximal action potential and increased the size of a maximal potential.

Neural Effects of Digitalis on Respiration

Early descriptions of pharmacological actions of digitalis also refer to effects on respiration (Traube, 1851). Gross (1914) first suggested that cardiac glycosides act directly on the brain stem respiratory center. More recently Cameron (1967) described hyperventilation in the rabbit in response to injections of ouabain into the lateral ventricles. He found that during this hyperventilation, cerebrospinal fluid was alkalotic and contained increased concentrations of K^+. He attributed the changes in respiration to changes in ionic constituents, particularly K^+, in the extracellular fluid surrounding respiratory neurons.

Digitalis also might stimulate respiration by increasing H^+ concentration. Leusen (1954) showed that an increased H^+ concentration in the brain stimulates respiration and Loeschcke and Koepchen (1958) attributed the H^+ effect to a central chemoreceptor

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stimulation. However, Yen and Chow (1974) showed that during hyperventilation induced by intravenous ouabain, CSF concentrations of H^+ decreased and K^+ concentration did not change. An action of cardiac glycosides to alter blood gases or pH could also enhance respiratory activity, perhaps via chemoreceptor stimulation. However, Sohn *et al.* (1970) and Yen and Chow (1974) described ouabain-induced respiratory enhancement occurring in cats and dogs with low pCO_2, high pH and normal pO_2. This suggested that the effects of digitalis on respiration were related to direct drug effects on neural structures.

Gillis (1972) described increases in phrenic nerve activity evoked by ouabain in the cat. Such increases in phrenic activity could have stemmed from drug actions in the central nervous system as suggested by Gross (1914). This view has also been presented by Sohn *et al.* (1970) and Gillis *et al.* (1972). However, it is equally possible that digitalis enhances respiration by increasing excitatory afferent input to central respiratory neurons. Digitalis has been shown to excite chemoreceptors (Schmitt *et al.*, 1958a,b) and the excitatory influence of peripheral chemoreceptors on respiration is well known (see reviews by Dejours, 1962; Biscoe, 1971). Digitalis could also activate other excitatory pressoreceptor afferents from the lung (Larrabee and Knowlton, 1946; Reynolds, 1962) and from intercostal muscles (Decima *et al.*, 1969).

Effects of Digitalis on Na^+-K^+-ATPase

For the past twenty years an area of active research has centered around the active transport of Na^+ in nervous tissue, the

enzymes involved in this transport, and the drugs which inhibit both enzyme activity and Na^+ transport. Hodgkin and Keynes (1955) showed that active transport of Na^+ from the giant axons of cephalopods was markedly reduced by the action of metabolic inhibitors. Caldwell and Keynes (1959) demonstrated that ouabain likewise caused a sharp fall in Na^+ efflux from giant axons. In a study concerned with squid axon phosphorus metabolism, Caldwell (1960) showed that the effect of dinitrophenol and cyanide on Na^+ pumping metabolism was related to depletion of adenosine triphosphate (ATP). Ouabain apparently was effective by a mechanism unrelated to ATP depletion. Skou (1957, 1960) characterized an ATPase in crab leg nerves which he suggested was related to the transport of Na^+ and K^+ across the nerve membrane. This ATPase required the presence of Na^+, K^+ and Mg^{++} for its activation. In a review article Skou (1965) discussed the inhibitory action of cardiac glycosides on this Na^+-K^+-activated ATPase and further substantiated the relationship of the enzyme system to active transport of cations. The relationship between active Na^+ efflux, Na^+-K^+-ATPase and the ability of cardiac glycosides to inhibit active Na^+ efflux was documented again in giant axons by Bonting *et al.* (1962) and Baker *et al.* (1969).

Some suggestions have been made relating the inhibition of Na^+-K^+-ATPase to altered nervous excitability. Inhibition of Na^+-K^+-ATPase could induce depolarization in nerve membranes which are dependent upon the sodium pump for a portion of their polarity. The contribution of the sodium pump to the resting potential differs

in various species. In those in which the pump generates a portion of the polarity, digitalis might induce a considerable depolarization and cause the membrane to be more easily excited to threshold for an action potential. To generate polarity, the sodium pump must transfer three Na^+ ions out of the cell in conjunction with moving two or less K^+ ions into the cell. This results in a metabolically driven intracellular negativity and the mechanism is referred to as the electrogenic pumping of sodium. Keynes (1974) stated that

> there is now plenty of evidence (derived principally from studies of excitable tissues) of the operation of electrogenic pumps that extrude sodium ions and at the same time create an electrical potential difference across the membrane.

He also reasoned that such a pump could be electrically neutral or electrogenic depending on prevailing conditions. Kerkut and York (1971) have summarized the literature regarding possible contributions of the electrogenic pump to membrane potential as follows:

Cat spinal motoneuron	10 mV	(Coombs *et al.*, 1955a,b)
Mammalian C fibers	4 mV	(Ritchie and Straub, 1952)
Mammalian A fibers	35 mV	(Rang and Ritchie, 1968)
Helix (snail) nerve cells	30 mV	(Kerkut and Thomas, 1965)
Aplysia nerve cells	30 mV	(Carpenter and Alving, 1968)
Sepia (squid) giant axon	1.8 mV	(Hodgkin and Keynes, 1955)

If the sodium pump contributes 4, 10 or 35 mV to the membrane potential of mammalian nerves, digitalis could depolarize them to enhance excitability. Kerkut and Thomas (1965) and Carpenter and Alving (1968) both showed that ouabain or decreased K^+ in the bathing media of invertebrate axons blocked the electrogenic component of the membrane potential. Therefore, inhibiting the sodium pump

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(Na^+-K^+-ATPase) by ouabain or decreased extracellular K^+ concentrations caused the membrane to depolarize.

However, many investigators do not support the electrogenic pump theory. Ritchie and Straub (1957) suggested that the sodium pump is electrically neutral but described data in which ouabain depolarized nonmyelinated fibers in a sucrose gap preparation. They proposed that even if a neutral pump were inhibited, Na^+ would accumulate in the cell; but more critically, K^+ would travel down its concentration gradient, thus leaving the cell. The inhibited pump would not transport K^+ back inside the cell; thus, the ratio of K^+ outside to K^+ inside would increase. Since this ratio is the main determining factor of the value of the resting potential (Goldman, 1943), the membrane would depolarize. These conclusions were supported when ouabain had a greater effect on the resting potential in nerves with sheaths intact than in those which had been desheathed. The sheath provides an effective barrier to the diffusion of K^+ away from the axon, thus allowing K^+ to accumulate in the immediate area of the membrane as K^+ leaves the cell, causing a decrease in the K^+-concentration gradient.

The effects of Na^+-K^+-ATPase inhibition on nerve cell membranes is greater on the smaller C fibers than on the larger A and B fibers. Whether these effects are related to alterations in an electrogenic or a neutral Na^+ pump, the imbalance of Na^+ and K^+ within a smaller fiber caused by Na^+-K^+-ATPase inhibition would be greater than that in larger fibers. Ionic fluxes across the membrane vary with surface area; thus, an equivalent flux of Na^+ or K^+ per

cm^2 surface area would result in a greater concentration change within a small fiber than within a large fiber. Thus, C fibers are probably the first to have altered excitability following inhibition of Na^+-K^+-ATPase.

Cardiac glycosides affect many neural structures and their neurotransmitters such as acetylcholine (Birks, 1963) and norepinephrine (Dengler, 1962). Digitalis can also enhance both excitatory and inhibitory reflexes in the cat spinal cord (Osterberg and Raines, 1973). This suggests that the mechanism of action for these drugs involves a very basic mechanism of cell function, one which could influence all neural events. It is tempting to speculate that inhibition of the membrane enzyme Na^+-K^+-ATPase could be involved in the mechanism of action of digitalis since disruption of Na^+ and K^+ balances within neural cells could lead to changes in excitability. Although a causal relationship has not been proven, the strong correlation between inhibition of Na^+-K^+-ATPase and pharmacological actions of digitalis is well known. When doses of digitalis in an animal are adequate to evoke responses, such as enhanced inotropy in heart or natriuresis in the kidney, and then those organs are removed, the Na^+-K^+-ATPase measured is inhibited (Akera *et al.*, 1970; Hook, 1969). This relationship has also been observed in the brain. Donaldson *et al.* (1971) and Venturini and Palladini (1973) injected 10-100 µg of ouabain into the cerebral ventricles of rats and guinea pigs. This treatment caused hyperactivity or convulsions and brain Na^+-K^+-ATPase from these animals was inhibited 50-98%. Donaldson *et al.* (1972) also conducted a comparative study

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of the effectiveness of various cations in the inhibition of rat brain Na^+-K^+-ATPase. *In vitro* experiments showed that inorganic ions varied in efficacy (Zn > Cu > Fe > Mn) in the specific inhibition of Na^+-K^+-ATPase. Intraventricular injections of the same cations or ouabain resulted in convulsions. The seizure inducing potency of the cations followed the same order as their Na^+-K^+-ATPase inhibitory potency. Thus, perhaps pharmacological effects of digitalis on neural excitability can be detected by biochemical changes which remain in the neural tissue and can be measured *in vitro* after the tissue has been removed from the animal.

Specific Objectives

The relative effect of digitalis at central and peripheral neural sites has not been well defined. Four groups of experiments were conducted to identify the primary sites of sympathetic action of digitalis.

The increased sympathetic discharge to the heart and vasculature has been suggested to stem from direct drug actions on central sympathetic neurons. However, it is difficult to separate central effects from peripheral effects following intravenous administration of digitalis. In contrast, administration of a drug directly into the brain allows one to observe its central effect uncomplicated by peripheral influences. Direct effects of central injections of digitalis on peripheral sympathetic nerve activity have not been described. Thus, small volumes of the rapidly acting cardiac glycoside, ouabain, were injected into sympathetic sites in the cat medulla and hypothalamus to define alterations in sympathetic

nervous discharge caused by a central action of the drug. The medulla was chosen as one area for administration because of its well known importance in the genesis of spontaneous sympathetic tone (Alexander, 1946). Ouabain was injected into the hypothalamus because this area also influences cardiovascular function (Karplus and Kreidl, 1927; Fuster and Weinberg, 1960). Drug effects were monitored on the vasoconstrictor external carotid nerve (Bishop and Heinbecker, 1932), on preganglionic fibers entering the stellate ganglion, and on the cardioaccelerator inferior cardiac nerve (Bronk *et al.*, 1936).

The goal of the second group of experiments was to define the relative effects of intravenously administered digitalis in peripheral and central sites. Digoxin, a cardiac glycoside with an onset of action of 5-30 min, was administered intravenously to 4 groups of cats. Drug effects on the activity of preganglionic splanchnic nerves were compared to effects on postganglionic inferior cardiac nerves in the presence and absence of chemoreceptor and baroreceptor reflexes. These sympathetic nerves were selected since digitalis has been reported to alter their activity (McLain, 1969; Pace and Gillis, 1974).

To compare the pattern of effects of digitalis on sympathetic nerves with those on a nerve which is not of sympathetic origin, drug effects were also observed on phrenic nerve activity. This nerve was also selected because digitalis enhances its activity. To estimate the involvement of peripheral afferent influences in the action of digitalis on phrenic activity, effects of digoxin were first observed in cats with intact respiratory reflexes. Drug

effects in these cats were compared to effects in cats whose chemoreceptor and pressoreceptor influence on respiration had been interrupted.

The last group of experiments was designed to reveal any biochemical effect of digitalis in the brain which might suggest a central action of the drug. The concentrations of digoxin in cerebrospinal fluid were measured during slow administration of lethal doses of this drug. The effects of these concentrations of digoxin on the membrane enzyme Na^+-K^+-ATPase were determined *in vitro* in 8 brain areas of the cat. Another group of cats were treated with lipid-soluble digitoxin, which was administered slowly until the cats died in ventricular fibrillation. Activity of Na^+-K^+-ATPase was examined *in vitro* after the brains had been removed. Additionally, the magnitude of the binding of digitoxin to brain Na^+-K^+-ATPase was estimated from the ATP-dependent binding *in vitro* of (^{3}H)-ouabain to the brain homogenate. Tritiated-ouabain binding was used to estimate the concentration of Na^+-K^+-ATPase unoccupied by digitoxin. Control Na^+-K^+-ATPase activity and (^{3}H)-ouabain binding were determined in brain homogenate from cats which had been infused with saline.

METHODS

General Methods

Preparation of Animals. Cats were anesthetized with an intraperitoneal injection of a mixture of sodium diallylbarbiturate (50 mg/kg), urethane (200 mg/kg) and monoethyl urea (200 mg/kg) or with an intravenous injection of α chloralose (40 mg/kg). A femoral artery and vein were cannulated and a tracheostomy tube inserted. Animals were artificially respired with room air by a Harvard respiration pump which was adjusted to deliver a volume of 35-50 ml at a rate of 14-18 respirations per minute (RPM). The respiratory rate and volume varied with the size of the cat (1.5-3.0 kg) and approximated normal respiratory parameters for the anesthetized cat (Bartorelli and Gerola, 1963). Temperature was maintained at approximately 38°C with a thermostatically controlled circulating hot water heating pad.

Sympathetic or phrenic nerves were exposed, desheathed, tied and severed peripherally. The skin which had been excised to expose these nerves was retracted and elevated by attaching it with long sutures to a metal flexaframe structure surrounding the surgical exposure. This allowed the nerves to be immersed in a pool of warm mineral oil which was used to maintain viability of the nerves and to provide insulation at the recording site.

Data Acquisition. Blood pressure was monitored from a femoral artery and displayed on a Grass polygraph. Lead II electrocardiogram was continuously monitored and a Grass EKG tachograph preamplifier allowed heart rate to be determined periodically.

Biphasic electrical activity of the sympathetic or phrenic nerves was recorded with a bipolar platinum electrode. One terminal of the electrode was active while the other was grounded. Spontaneous activity was amplified by a Grass 7P3 or P511 AC preamplifier with a band width of 30-500 Hz and displayed on a Grass polygraph. A Grass 7P10B integrator provided cumulative integration of spontaneous activity. The sensitivity of the integrator was adjusted so that control nerve activity produced a 70-80 sec epoch. The integrator was then calibrated with a known AC signal to determine the total accumulated voltage with respect to time (μV·sec) necessary to reset the integration and complete an epoch.

Specific Methods

Effects of Centrally Administered Ouabain on Sympathetic Activity

Preparation of animals. Thirty-four cats were anesthetized with the dial-urethan mixture. All cats were placed in a stereotaxic instrument. In some animals the stereotaxic instrument was rotated 180° to facilitate a ventral surgical approach to the superior cervical ganglion. Portions of the esophagus and trachea were removed and the external carotid nerve, a branch of the superior cervical ganglion, was exposed and desheathed. The ninth

and tenth cranial nerves were sectioned at the jugular foramen to prevent baroreceptor and chemoreceptor afferent influences from reaching the brain stem and interfering with observations of the possible central effects of ouabain. The medulla oblongata was approached ventrally in these cats by removing a caudal portion of the base of the skull. The dura mater was opened without damage to underlying vertebral and basilar arteries.

The traditional stereotaxic position was maintained in the remainder of the animals. The ninth and tenth cranial nerves were also severed in these cats. Portions of the first and second ribs were removed extrapleurally to expose the right stellate ganglion. Preganglionic fibers entering the stellate ganglion or the postganglionic inferior cardiac nerve were desheathed and prepared for recording. Preganglionic nerves selected were approximately the same size as the postganglionic nerves. Removal of portions of occipital, parietal and frontal bone provided dorsal access to the hypothalamus and medulla.

Data acquisition. Spontaneous activity from these nerves was recorded and integrated as described in General Methods. Responses evoked by electrical stimulation of the medulla and hypothalamus were also recorded from these nerves. These evoked responses were signal averaged. Evoked activity was amplified by a Grass P511 preamplifier with a band width of 1-1000 Hz and then averaged with a PDP-8e Digital Equipment Corporation computer and displayed on an XY-recorder. The signal-averaging technique sums activity time-locked to the stimulus while spontaneous activity and

interference signals are averaged out to approach a straight line (Figure 1). Thus, the signal to noise ratio improves in proportion to the square root of the number of trials taken. Averaged responses are less variable than single responses. Thus, drug effects on the averaged response are less ambiguous than effects on a single response.

Electrode design and placement. A 3-inch 30-gauge needle was insulated to within 0.5 mm of its tip and used stereotaxically both as a monopolar stimulating electrode and as a means of delivering drug into the brain. Stimuli from a Grass S48 stimulator were passed through a capacitance-coupled isolation unit and applied to brain sites through the needle electrode. Stimulus parameters were 3-10 V, 0.5 msec, and 1-50 Hz. Since differing responses were obtained when these electrodes were advanced 1 mm through the brain, stimulus current was probably minimal beyond a radius of 1.0 mm. A David Kopf microinjection unit was used to deliver 1 to 2 µl volumes of drug or control solutions.

Electrodes were placed in the hypothalamus and medulla from a dorsal approach in those animals in which nerve activity was recorded from fibers entering or leaving the stellate ganglion. Standard stereotaxic techniques were utilized in these animals following the coordinates of Snider and Neimer (1970).

The medulla was approached ventrally in cats in which activity was recorded from the external carotid nerve. The ventral median fissure was used as a midline reference for mediolateral

Figure 1. Signal averaging of an evoked response.

The top row is a response from the external carotid nerve to a single shock in the medulla. The second row is an average of 30 responses. Signal averaging sums the early activity time locked to the stimulus while the later spontaneous activity and interference signals average out approaching a straight line. The signal to noise ratio improves in proportion to the square root of the number of trials taken. The averaged response (lower row) is much less variable than a single response (top row).

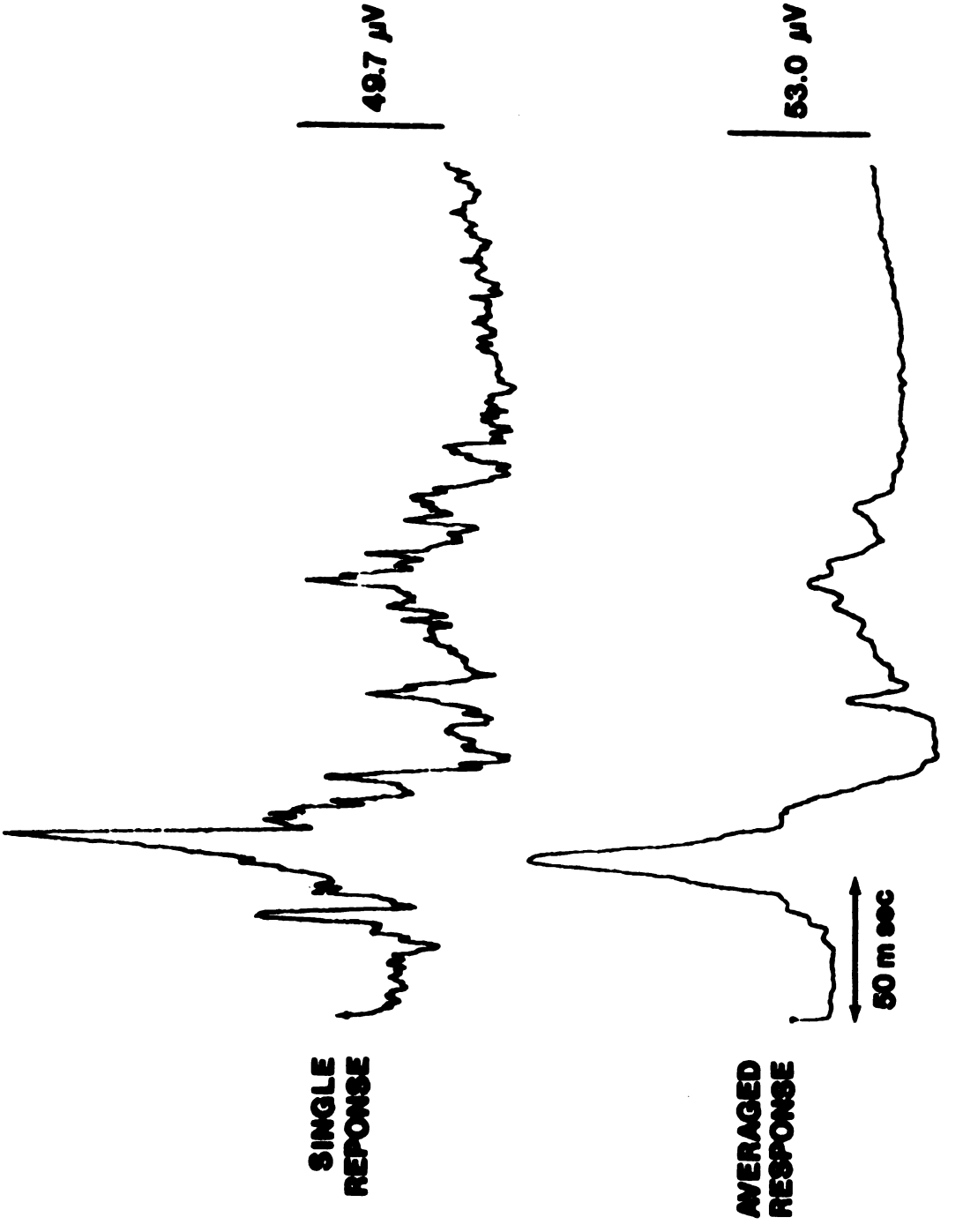

Figure 1

orientation and the ventral surface of the brain provided a reference for dorsoventral orientation.

The medullary sites tested were lateral 2-3 mm from midline and 0-4 mm rostral to the obex. Ouabain was injected into either dorsal or ventral areas. Ouabain was also injected into the hypothalamus at the level of the mammillary bodies approximately 2.5 mm lateral from midline. Anatomical location of electrode track after the termination of an experiment was accomplished grossly in some cats and histologically in others.

Identification of site of injection. High frequency stimulation (50 Hz) lasting approximately 10 sec was applied to medullary or hypothalamic sites until one was located which responded by producing an increased heart rate, increased blood pressure, and bursting increases in nerve activity (Figure 2). The minimum criteria for site selection in either area were a 4-fold increase in nerve activity and a 20 beat per min increase in heart rate or a 75 mmHg increase in blood pressure. Single shocks (1 Hz) applied to the medulla or hypothalamus also evoked responses as shown in the lowest panel of Figure 2. The 128 brain sites selected thus had approximately equivalent excitatory influence on activity of all three sympathetic nerves and this influence was sufficient to alter heart rate and blood pressure. In 24% of the selected sites, electrical stimulation evoked cardiac arrhythmias. When recording from vasoconstrictor nerves, blood pressure changes were considered the primary cardiovascular criterion for site selection. Similarly, when recording from cardioaccelerator nerves,

Figure 2. Criteria used to define typical medullary or hypothalamic injection site.

Upper 3 panels: simultaneous cardiovascular and external carotid nerve responses to high frequency electrical stimulation of the medulla. Stimulus parameters were 6 V, 0.5 msec, 50 Hz. The bar on the time marker indicates the period of stimulation. Lower panel: response of the same nerve to stimulation of the same medullary site at 1 Hz.

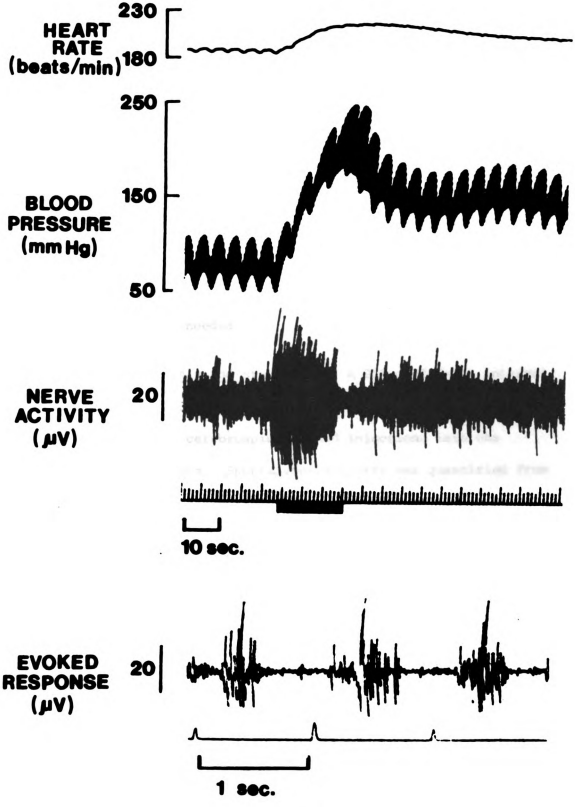
HEART RATE (beats/min)
230
180
BLOOD PRESSURE (mm Hg)
250
150
50
NERVE ACTIVITY (μV)
20
10 sec.
EVOKED RESPONSE (μV)
20
1 sec.

Figure 2

tachycardia was the cardiovascular parameter considered most important in site identification.

Drugs. Ouabain octahydrate (Sigma Chemical Company) was dissolved in either saline or artificial cerebrospinal fluid prepared with the constituents described by Davson (1967). Both vehicles were adjusted to pH 7.4. Ouabain concentrations ranged from 0.5-2000 ng/µl.

Animals were immobilized with gallamine triethiodide (4 mg/kg) to prevent somatomotor responses to electrical stimulation of the brain. Supplemental doses of gallamine triethiodide (1-2 mg/kg) were administered as needed.

Data analysis and statistics. A 95% confidence interval (Sokal and Rohlf, 1969) was constructed from the nerve responses to control saline or cerebrospinal fluid injections into the medulla or hypothalamus. Spontaneous activity was quantified from the cumulative integration epoch time intervals and evoked potentials were measured at their peak. Nervous responses to ouabain exceeding the limits of the confidence interval were considered to be significantly different from pre-drug activity. Each response to ouabain from all of the animals was compared to the confidence interval and classified as an increase or a decrease in activity or no change. Then the data from all cats were grouped according to these classifications and a mean percent change from control $\pm$ SEM was calculated for each group. The Student's t test and the Fisher F ratio test were used in some instances to justify pooling of

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control responses to saline or CSF. The level of statistical significance was set at $P<.05$. Datawere expressed as mean percent change from pre-drug activity $\pm$ standard error.

Effects of Intravenously Administered Digoxin on Sympathetic Activity

Preparation of animals. Twenty-five cats were anesthetized with α chloralose. In 13 cats, the left preganglionic splanchnic nerve was exposed retroperitoneally, desheathed, tied, and severed proximal to its entry into the celiac ganglion. In 12 cats, the right stellate ganglion was exposed by removing portions of the first and second ribs extrapleurally. Then the inferior cardiac nerve, a branch of the stellate ganglion, was desheathed, tied and severed 2-3 mm distal to the ganglion. The ninth and tenth cranial nerves were sectioned at the jugular foramen in half the cats in each group.

Data acquisition. Spontaneous activity of the sympathetic nerves was recorded and integrated as described in General Methods. Arterial blood pH, pO_2 and pCO_2 were measured with an Instrumentation Laboratory or a Radiometer blood gas analyzer at 30-60 min intervals as needed to maintain normal respiratory and acid-base balance. The respiratory volume and rate were adjusted to maintain arterial pO_2 and pCO_2 constant within normal limits.

Drugs. Sodium bicarbonate was infused intravenously if needed to maintain pH between 7.35 and 7.45. Animals were immobilized

with gallamine triethiodide (4 mg/kg) to prevent emetic responses to digoxin. Supplemental doses of gallamine triethiodide (1-2 mg/kg) were administered as needed. After nerve activity had been stabile for 30 min, 20 µg/kg of digoxin (Lanoxin, Burroughs Wellcome) was injected intravenously every 15 min until the cat died. The last dose, which was usually given during severe arrhythmia, was sometimes less than 20 µg/kg if fibrillation appeared imminent. After the onset of arrhythmia, volume expansion with intravenous dextran was often initiated in an attempt to maintain constant blood pressure.

Data analysis and statistics. Spontaneous nerve activity was quantified by measuring the cumulative integration epoch time intervals. A pre-drug initial epoch time and epoch times approximately 10 min after each dose of digoxin were measured. The last data measurements were made immediately preceding ventricular fibrillation. Nerve activity was expressed as a percent of pre-drug initial activity calculated from the measured epoch time intervals. Data were expressed as means $\pm$ standard error. Random complete block factorial analyses were used to statistically verify treatment effects and least significant difference tests were used to compare means (Sokol and Rohlf, 1969). The criterion for statistical significance was $P<.05$.

Effects of Intravenously Administered Digoxin on Phrenic Nerve Activity

Preparation of animals. Twenty-six cats were anesthetized with α chloralose. In all the cats, the right phrenic nerve was exposed in the lower cervical region. It was then desheathed, tied and severed. The ninth and tenth cranial nerves were sectioned at the jugular foramen in 18 of these cats. Pneumothoracotomies were performed in the cats whose ninth and tenth cranial nerves were severed. In 5 cats a loop of suture was atraumatically passed around both nerves at the level of the jugular foramen. This suture was tied near the end of these experiments to interrupt afferent influence on phrenic nerve activity.

Data acquisition. Spontaneous phrenic nerve activity was recorded and integrated as described in General Methods. Arterial blood pH, pO_2 and pCO_2 were measured with an Instrumentation Laboratory or a Radiometer blood gas analyzer as needed to maintain normal respiratory and acid-base balance. Expired CO_2 was monitored with a Beckman LB-2 Medical Gas Analyzer which had been calibrated with gases having known CO_2 concentrations. Expired air was sampled from a fine cannula inserted to the bifurcation of the trachea through a side arm of the tracheostomy tube. Subtle adjustments in respiratory rate (0.2-0.5 RPM) were made during the experiments to maintain expired CO_2 and pCO_2 constant. Changes in expired CO_2 closely parallel changes in pCO_2 (Hartsfield, 1973). The continuous end-tidal CO_2 monitor detected changes in CO_2 as little as 0.8 mmHg.

This allowed compensatory adjustment in respiratory parameters to be made quickly, often before phrenic activity had begun to change. The respiratory volumes and rates maintained pCO_2 and pO_2 within normal limits (Hartsfield, 1973). Blood pCO_2 varied between 25-40 mmHg and pO_2 between 80-110 mmHg. Arterial blood pH was maintained between 7.35 and 7.45 by infusions of sodium bicarbonate when necessary. Blood pH varied less than 0.1 unit during an experiment.

Drugs. Animals were immobilized with gallamine triethiodide (4 mg/kg) to facilitate artificial respiration and to prevent emetic responses to digoxin. Supplemental doses of gallamine triethiodide (1-2 mg/kg) were administered as needed. After nerve activity had been stabile for 30 min and blood pH, pCO_2 and pO_2 were within normal limits, 20 µg/kg digoxin ("Lanoxin", Burroughs Wellcome) was injected intravenously every 15 min until the cat died.

Data analysis and statistics. Data were analyzed as described in the sympathetic nerve recording experiments. The statistical methods were also the same with the exception that completely random factorial analyses were used in the phrenic experiments. A complete block design could not be utilized since one cat died at a much lower dose than the rest of the animals; thus, the replications for the early and late doses of digoxin were unequal.

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Concentrations of Digitalis in the Central Nervous System Following Intravenous Administration and Their Effects on Na^+-K^+-ATPase

Experimental protocol. Twenty-three cats were anesthetized with the dial-urethan mixture. In 9 cats the skin and muscle overlying the foramen magnum were incised and retracted. A 27-gauge needle with fine polyethylene tubing attached was punctured through the dura into the CSF and sutured in place. Bloodless CSF samples were obtained through this needle and tubing. Both femoral veins were cannulated. Radiolabeled digoxin (20 µg/kg) was injected every 15 min into one vein until the cats died in arrhythmia. Blood samples were drawn from the other femoral vein. To prevent emetic responses to digoxin, these cats were immobilized with gallamine triethiodide as described earlier. Randomly labeled (^3H)-digoxin (New England Nuclear) with specific activity approximately 9 Ci/mmole was diluted 1:25 with unlabeled digoxin (Burroughs Wellcome) and used in 7 cats. Specifically labeled (12α-^3H)-digoxin with specific activity 18.3 Ci/mmole was diluted 1:50 with unlabeled digoxin and administered to 2 cats. A control blood and CSF sample were taken and then samples were taken every 30 min after the onset of drug administration until the last samples were taken at death.

After anesthesia 4 cats were killed with an air embolus and their brains quickly removed. Approximately 500 mg of tissue was sampled from 8 areas, including posterior medulla, anterior medulla, midbrain, thalamus including hypothalamus, preoptic area, medial occipital cortex, pyriform area including amygdaloid body, and cerebellum. The tissue was quickly frozen. These areas were

selected since they all contain or influence central sympathetic neurons (Crosby, 1962). The thalamic region was also chosen because it contains higher concentrations of digitalis than other brain areas after central or intravenous drug administration (Dutta and Marks, 1966; Donaldson *et al.*, 1971).

Six cats were infused with digitoxin ("Crystodigin", Eli Lilly Company) at a rate of 1.0-1.5 µg/kg/min until they died in arrhythmias at a dose of approximately 180 µg/kg. These cats were also paralyzed with gallamine triethiodide. Four more cats were infused with saline for an equivalent time period (2 hr) and were killed with an air embolus. Brains were removed and the same 8 areas described above were sampled and frozen.

Biochemical analyses. Blood samples from the cats treated with (^{3}H)-digoxin were allowed to clot and serum was aspirated. Serum (1.5-2.0 ml) was placed in a Millipore ultrafiltration chamber and forced with nitrogen gas (25 psi) through a filter having a pore size admitting particles with molecular weights less than 25,000. With this technique 100 µl of filtrate containing free (non-protein bound) digoxin was collected in approximately 10 min. CSF (200 µl) and serum filtrate (100 µl) containing free digoxin were placed in counting vials containing 15 ml PCS scintillation solution (Amersham/ Searle) and placed in the dark for 12 hrs. Tritium in these samples was counted for 20 min in a Beckman LS-100 liquid scintillation spectrometer. A sample of serum (20 µl) containing both bound and free drug was digested for 12 hrs with 200 µl of Soluene (Packard Instrument Company) in a counting vial. Then 15 ml of

scintillation cocktail (4 g 2,diphenyloxazole (PPO) + 167 mg p-bis-[2-(5-phenyloxazolyl)]-benzene (POPOP) + 1 l toluene) and 2 ml of ethyleneglycol monomethylether ("Piersolve", Pierce) were added. These samples were also dark adapted for 12 hrs and then counted. Cerebrospinal fluid and plasma filtrate samples and aliquots of digoxin solution were evaporated in counting vials and then reconstituted with 500 µl ethanol benzene (1:9) and 200 µl water (CSF) or 100 µl water (serum filtrate). These samples were then treated like the unevaporated samples.

The brain samples used in the *in vitro* inhibition study were homogenized in a solution containing 0.25 M sucrose, 5.0 mM histidine, 5.0 mM EDTA and 0.15% Na-deoxycholate at pH 7.5 to make a 10% homogenate. A Potter-type homogenizer with a motor driven Teflon pestle was used. The homogenate was centrifuged at 100,000 g for 30 min using a Beckman L-350 preparative ultracentrifuge. The pellet was resuspended in 4.5 ml of the same homogenizing solution (with 0.1% Na-deoxycholate) with a T-pestle in a Dounce homogenizer and centrifuged again at 100,000 g for 30 min. The pellet was suspended in 5.0 ml of resuspending solution (.25 M sucrose, 1 mM EDTA, 5 mM histidine and pH 7.5). A small amount of the resuspended particulate fraction was diluted 1:60 with water and assayed for protein by the method of Lowry *et al.* (1951). The protein concentration was adjusted to 0.5 mg protein/ml with resuspending solution. Na^{+}-K^{+}-ATPase and Mg^{++}-ATPase activity were assayed in the presence of 3×10^{-9}, 1×10^{-8}, 3×10^{-8} and 1×10^{-7} M digoxin. The ATPase reaction was initiated with the addition of KCl according to the method of Akera (1971).

A very simple technique was used to prepare the brain samples from the cats treated with digitoxin or saline. Approximately 500 mg of brain tissue was homogenized with resuspending solution (0.25 M sucrose, 1.0 mM EDTA, 5.0 mM histidine, pH 7.5) to make a 10% homogenate. The same homogenizer described above was used. In these experiments Na^+-K^+-ATPase was assayed by the method of Akera (1971). Here the ATPase reaction was initiated by the addition of ATP. ATP dependent binding of (^3H)-ouabain to a 0.5% homogenate of the same tissue was measured by the method of Akera *et al.* (1973).

Data analysis and statistics. Data were expressed as means $\pm$ S.E.M. Group comparisons were made with a Student's t test (Sokal and Rohlf, 1969). Linear regression by the method of least squares was used to express the relationship between *in vitro* Na^+-K^+-ATPase inhibition and digoxin concentration. $P<0.05$ was the criterion for statistical significance.

RESULTS

Effects of Centrally Administered Ouabain on Sympathetic Activity

Medullary Microinjections

Effects on vasoconstrictor external carotid nerve activity. After identifying a medullary vasoconstrictor site, 2 µl of saline or ouabain dissolved in saline were injected into the area through the needle electrode without moving it. As shown in panels A and B of Figure 3, saline produced no apparent effect on spontaneous external carotid nerve activity. The change (quantified from the cumulative integration) produced by saline in panel A is a 7% decrease and in B it is a 5% increase. This indicated that the injection volume itself caused no apparent response. However, microinjections of ouabain caused marked changes in nerve activity. Increases of 20%, 38% and 129% in spontaneous activity in response to 1, 10 and 100 ng of ouabain, respectively, are shown in Figure 3, panel A. These are responses from three different vasoconstrictor sites in the same cat. In contrast, in another cat, 1 and 10 ng of ouabain produced reductions (28% and 37% decreases, respectively) in spontaneous activity (Figure 2, panel B). Again, each dose was injected into a different vasoconstrictor site.

Figure 3. Effects of 2 µl injections of saline or ouabain on activity of the external carotid nerve.

Panel A: effects of injections into 4 different medullary vasoconstrictor sites in the same cat. Saline had no apparent effect (a 7% decrease) on spontaneous discharge while each dose of ouabain increased activity. Twenty percent, 38% and 129% increases were produced by 1, 10 and 100 ng of ouabain, respectively. Panel B: effects of injections into 3 sites in another cat. Again saline had no apparent effect (5% increase). Here 1 ng of ouabain decreased activity 28% and 10 ng decreased it 37%. Percent change was calculated from cumulative integration records of activity.

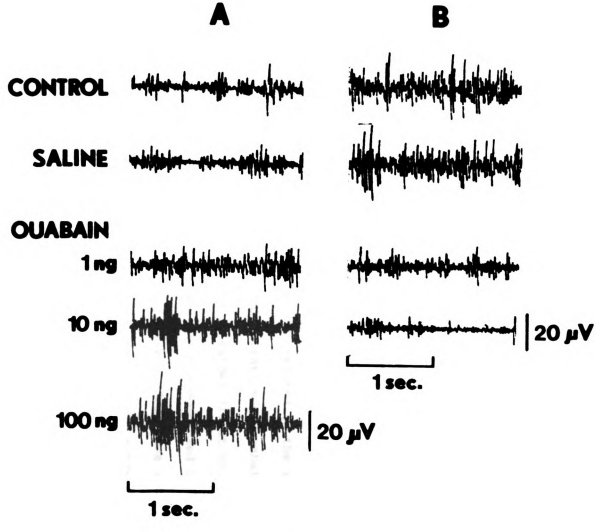
A
B
CONTROL
SALINE
OUABAIN
1 ng
10 ng
100 ng
20 μV
1 sec.
20 μV
1 sec.

Figure 3

Drug-induced changes usually took 1 to 2 min to develop and the maximum effect always occurred within 13 min of injection. Frequently, the maximum change lasted 5 to 10 min and then activity took 15 to 20 min to gradually return to the pre-drug level. The time course of drug action in a few sites was short, with the entire effect disappearing within 15 min of injection. After the large doses (100 and 1000 ng) the drug effect sometimes lasted longer, occasionally taking 40 to 60 min to return to pre-drug activity. Inhibitory effects of ouabain were sometimes preceded by a brief period of stimulation which occurred seconds after injection and lasted approximately 1 min. With this exception, no biphasic responses to ouabain occurred. The direction of the drug effect also was not related to the initial level of spontaneous nerve activity. Administration of ouabain to many sites produced no change in activity in either direction. Increases in blood pressure and heart rate often accompanied increases in nervous discharge evoked by ouabain. Similarly, decreases in these parameters paralleled ouabain-induced decreases in nerve activity. These cardiovascular responses occurred when either vasoconstrictor or cardioaccelerator nerve activity was monitored. The heart rate and blood pressure changes appeared independent of each other; sometimes one occurred and not the other and the magnitudes of heart rate and blood pressure changes were not always similar.

The dose range illustrated in Figure 3 includes 1 ng, the threshold dose, through 100 ng which produced large responses. One thousand ng (not shown) was the peak of the dose response curve. Nerve activity responses were quantified at each dose from 1-1000 ng.

They were expressed as percent change from pre-drug activity as calculated from the cumulative integration records. A 95% confidence interval was constructed from responses of the external carotid nerve to medullary injections of saline. Ouabain-induced responses of insufficient magnitude to extend beyond the confidence interval limits were classified as no response. Those extending beyond the confidence interval were classified, according to the direction of response, as increases or decreases. The mean responses to each dose at three time intervals after injection are presented in Table 1. No definitive dose response relationship could be established from this tabulated data. Although there was a tendency for drug-induced increases to become larger with increasing doses at the 1-3 min interval, this relationship was not apparent at the later times of measurement. However, the ouabain-induced inhibitions did display a trend toward a greater magnitude of response with increasing doses at all 3 time intervals. Also, the incidence of significant nervous responses to ouabain became greater as the dose increased. The ratio of significant responses to total trials was 18/38 (47%) at 1 ng, 20/44 (45%) at 10 ng, 39/54 (72%) at 100 ng and 24/27 (89%) at 1000 ng.

Extending the dose to 20 µg and the injection volume to 10 µl always produced a profound, long lasting decrease in nerve activity. Following these injections, the medullary site became completely refractory to electrical stimulation and no further blood pressure, heart rate or nerve activity responses could be elicited from that site.

Table 1. Effects of injections of ouabain into medullary vasoconstrictor sites on spontaneous activity of the external carotid nerve

Time after injection		Dose of Ouabain			
		1 ng	10 ng	100 ng	1000 ng
1-3 min	no change[a]	0.9 ± 1.3(5)[b,c]	-0.4 ± 1.5(7)	-0.6 ± 0.6(6)	-0.5 ± 0 (1)
	increase	21.8 ± 9.0(5)	15.6 ± 5.5(5)	65.3 ± 31.3(4)	57.5 ± 33.0(3)
	decrease	-12.6 ± 1.3(5)	-17.0 ± 3.0(3)	-22.8 ± 3.3(8)	-28.3 ± 2.8(5)
4-8 min	no change	0.4 ± 0.4(7)	3.4 ± 1.2(7)	0.0 ± 0.0(5)	0.0 ± 0.0(1)
	increase	38.5 ±18.5(2)	22.3 ± 6.1(6)	44.8 ± 7.3(2)	35.7 ± 17.8(3)
	decrease	-9.0 ± 3.2(3)	-17.5 ± 2.5(2)	-23.1 ± 4.6(11)	-25.3 ± 5.0(5)
9-13 min	no change	4.2 ± 1.6(8)	5.8 ± 0.9(10)	0.0 ± 0.0(4)	0.0 ± 0.0(1)
	increase	46.6 ± 0.0(1)	19.4 ± 4.0(2)	19.2 ± 3.3(3)	38.3 ± 15.9(3)
	decrease	-9.4 ± 1.7(2)	-16.3 ± 3.8(2)	-19.7 ± 4.0(11)	-23.3 ± 9.2(5)

[a]Classification of response as described in text.

[b]External carotid nerve activity was quantified from cumulative integration and expressed as mean percent change from pre-drug activity ± SEM.

[c]Numbers in parentheses indicate number of responses contributing to each mean percent change.

The mean magnitude of the three different responses of spontaneous external carotid nerve activity to medullary injections of ouabain are illustrated in the upper half of Figure 4. Responses to all doses (1-1000 ng) were pooled and grouped into time intervals following the injection. Again, the responses were grouped into those not significantly different from responses to saline and thus not extending beyond the confidence limits, and into those which were either greater than or less than pre-drug activity.

At all three time intervals a considerable number of injections produced no effect. However, when the responses which extended beyond the limits of the confidence interval were separately grouped into increases and decreases, the mean increase and the mean decrease at each time interval extended well beyond the limits of the confidence interval. It thus appears that when the ouabain injection evoked a change in nerve activity, it produced either a marked increase or a marked decrease. At the two later sampling times, the number of decreases in activity evoked by ouabain exceeded the number of increases.

Thus, the administration of ouabain to central sympathetic neurons produced either no change or an array of increases and decreases in spontaneous activity of a sympathetic nerve. The direction of the response also did not appear to relate to the dose since all effects were elicited at each dose from 1-1000 ng. However, spontaneous discharge on a sympathetic nerve is the product of outflow from a large area of the brain (Alexander, 1946), perhaps including supramedullary areas (Manning, 1965). Thus, an injection

Figure 4. Quantified effects of injections of ouabain into medullary vasoconstrictor sites on activity of the external carotid nerve.

Numbers on the ordinate are nerve activity responses to ouabain expressed as a percent change from pre-drug activity. Percent change was calculated from cumulative integration records of spontaneous activity or from the measured peak of the averaged evoked potential. Responses to all doses of ouabain (1-1000 ng) were pooled in this figure. Numbers on the abscissa are minutes after injection. Data were sampled at 3 time intervals. The stippled areas represent 95% confidence intervals constructed from nerve responses to injections of saline. Each response to ouabain was compared to the confidence interval and classified as an increase in activity, a decrease, or no change. Then the data from all cats were grouped according to these classifications and a mean percent change $\pm$ SEM was calculated for each group. The bars within the confidence interval indicate the mean nonsignificant responses; those extending beyond the interval are mean increases and mean decreases. The numbers in parentheses are the number of responses contributing to each group.

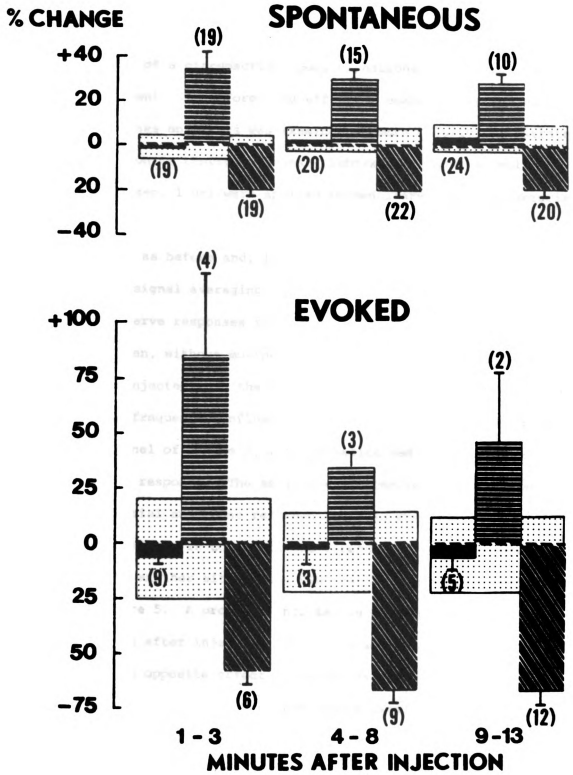
% CHANGE
SPONTANEOUS
+40
20
0
20
−40
(19)
(19)
(19)
(15)
(20)
(22)
(10)
(24)
(20)
EVOKED
+100
75
50
25
0
25
50
−75
(4)
(9)
(6)
(3)
(3)
(9)
(2)
(5)
(12)
1 - 3
4 - 8
9-13
MINUTES AFTER INJECTION

Figure 4

of 2 µl probably affected only a portion of the input to any one nerve. In contrast, a discharge evoked by a stimulus is the result of excitation of a circumscribed pool of neurons reached by the stimulus current. Therefore, the effect of ouabain on stimulus evoked responses on nerves was examined to more clearly demonstrate the drug action on central neurons. Submaximal single stimuli (2-8 V, 0.5 msec, 1 Hz) were applied to medullary sites characterized as described earlier (Figure 2). External carotid nerve activity was displayed as before and, in addition, directed into a PDP 8/e computer for signal averaging of evoked responses.

Thirty nerve responses to medullary single stimuli were signal-averaged. Then, without moving the electrode, saline or 100 ng of ouabain was injected into the same site. The dose 100 ng was chosen since it had frequently influenced spontaneous activity. As shown in the top panel of Figure 5, 2 µl of saline had no apparent effect on the evoked response. The amplitude and configuration of the initial potential and those recorded 2 and 15 min after injection were very similar. In contrast, the effect of 100 ng of ouabain injected into another site in the same cat is shown in the middle panel of Figure 5. A profound increase in the evoked response occurred 2 min after injection and 15 min later it was still increased. An opposite effect of microinjections of ouabain into a site in another cat is shown in the bottom panel of Figure 5 (note the increased amplification). Here ouabain dramatically reduced the magnitude of the evoked response 2 min after injection. At 15 min, it was equally reduced. In many experiments, evoked potentials were

Figure 5. Effects of injections of ouabain into medullary vasoconstrictor sites on signal-averaged evoked potentials in the external carotid nerve.

Each potential is the average of 30 responses to single shocks applied to the same medullary site. The top panel shows that 2 µl of saline injected into a vasoconstrictor site had no discernible effect on the potential evoked from that site. Stimulus parameters were 6 V, 0.5 msec, 1 Hz. The middle panel illustrates the effects of ouabain (100 ng) on a potential evoked from another vasoconstrictor site in the same cat. Ouabain increased the amplitude of this potential. Stimulus parameters were 8 V, 0.5 msec, 1 Hz. The lower panel shows an inhibitory effect of ouabain (100 ng) on an evoked potential from a vasoconstrictor site in another cat. Note the increased amplification. Stimulus parameters were 4 V, 0.5 msec, 1 Hz.

enhanced by ouabain in one site and inhibited in another site in the same animal. In addition, many averaged responses were left unchanged by ouabain injections. Drug effects on evoked activity had a similar time course to effects on spontaneous activity. An initial enhancement lasting 1 min occasionally preceded inhibition of evoked potentials but no other biphasic response pattern was observed. The direction of the response did not relate to the initial amplitude or configuration of the evoked potential.

Thus, as shown in the lower half of Figure 4, ouabain effects on evoked activity were qualitatively similar to those on spontaneous activity. In more than half of the trials, ouabain-induced increases and decreases in the amplitude of evoked potentials extended far beyond the confidence limits and again decreases occurred more frequently than increases. However, ouabain-induced changes in evoked potentials were greater in magnitude than changes in spontaneous activity. Furthermore, the mean response to saline showed greater variability in evoked activity than in spontaneous activity, as indicated by the larger confidence interval.

Effects on cardioaccelerator sympathetic nerve activity. Following the same experimental protocol, the effects of microinjections of ouabain into medullary cardioaccelerator sites were observed on sympathetic innervation to the heart. The effects of saline injections on spontaneous and evoked activity of stellate preganglionic and postganglionic nerves were very similar to the effects on the external carotid nerve. No statistical difference between the 3 groups of responses to saline was detected by the Fisher F ratio and

Student's t test. Thus, these data were pooled and the same confidence interval used for the activity from all three nerves.

Effects of ouabain injected into the medulla on the activity of stellate preganglionic nerves were qualitatively similar to the effects on external carotid nerve activity. As shown in Figure 6, both increases and decreases in spontaneous and evoked stellate preganglionic activity were produced by 100 ng of ouabain. Again, decreases in both spontaneous and evoked activity were more frequent than increases. Significant changes were seen in more than half the trials. However, in some sites ouabain produced no significant effect on the peripheral nerve activity. The small magnitude of change in spontaneous activity produced by medullary injections of ouabain may have been due to the low initial basal activity which is characteristic of the stellate preganglionic nerve. In contrast, effects of ouabain on evoked responses in this nerve extended considerably beyond the confidence limits.

When recording from the postganglionic inferior cardiac nerve, a different trend in the pattern of responses to medullary injection of ouabain appeared. As shown in Figure 7, inhibition of spontaneous and evoked activity became very dominant. With the exception of one trial in which an increase in spontaneous activity occurred, 100 ng of ouabain inhibited activity in the inferior cardiac nerve in all medullary sites which responded significantly. Following the pattern of the other two nerves described, ouabain changed the evoked response to a greater extent than it affected spontaneous activity, and injections into some sites had no effect.

Figure 6. Quantified effects of injections of 100 ng ouabain into medullary cardioaccelerator sites on activity of stellate preganglionic nerve.

Format is the same as described for Figure 4.

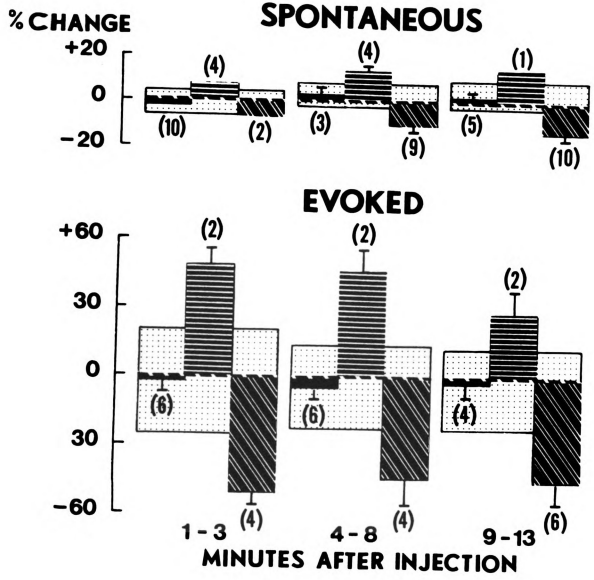
% CHANGE
SPONTANEOUS
+20
0
-20
(4)
(10)
(2)
(4)
(3)
(9)
(1)
(5)
(10)
EVOKED
+60
30
0
30
-60
(2)
(6)
(4)
(2)
(6)
(4)
(2)
(4)
(6)
1 - 3
4 - 8
9 - 13
MINUTES AFTER INJECTION

Figure 6

Figure 7. Quantified effects of injections of 100 ng ouabain into medullary cardio-accelerator sites on activity of inferior cardiac nerve.

Format is the same as described for Figure 4.

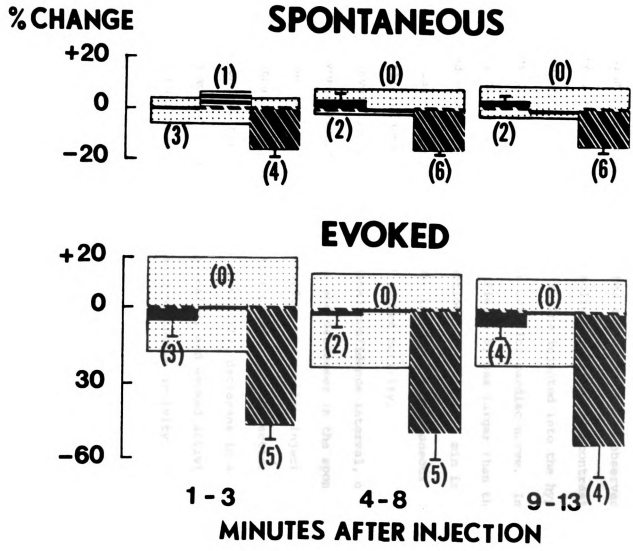
% CHANGE
SPONTANEOUS
+20
0
-20
(1)
(0)
(0)
(3)
(2)
(2)
(4)
(6)
(6)
EVOKED
+20
0
30
-60
(0)
(0)
(0)
(3)
(2)
(4)
(5)
(5)
(4)
1 - 3
4 - 8
9 - 13
MINUTES AFTER INJECTION

Figure 7

Hypothalamic Microinjections

Effects on cardioaccelerator sympathetic nerve activity.

Ouabain (100 ng dissolved in 2 µl saline) was also injected into hypothalamic cardioaccelerator sites and effects were observed on stellate preganglionic and postganglionic nerves. In contrast to the medullary injections, 2 µl of saline injected into the hypothalamus produced responses on the inferior cardiac nerve. In general, the confidence interval (Figure 8) was larger than that produced by medullary saline injections. At the 9-13 min interval 2 µl of saline produced an apparent increase in spontaneous activity and at all intervals it inhibited evoked activity.

However, in spite of the widened confidence interval, ouabain still evoked significant increases and decreases in the spontaneous and evoked activity of inferior cardiac nerves when injected into the hypothalamus. Like the effects of medullary injections of ouabain on all nerves described previously, decreases in activity occurred more frequently than increases, and evoked activity was affected to a greater extent than spontaneous activity. Some evoked responses were not altered by hypothalamic injections and spontaneous activity was often not changed. The lack of effect on spontaneous activity may have been due to the minor influence of the hypothalamus on sympathetic tone in the resting state.

When activity on stellate preganglionic nerves was observed, two alterations in experimental protocol were made in an attempt to reduce the responses to control injections. Instead of saline, artificial cerebrospinal fluid was used as a vehicle and the injection

Figure 8. Quantified effects of injections of 100 ng ouabain into hypothalamic cardioaccelerator sites on activity of inferior cardiac nerve.

Format is the same as described for Figure 4.

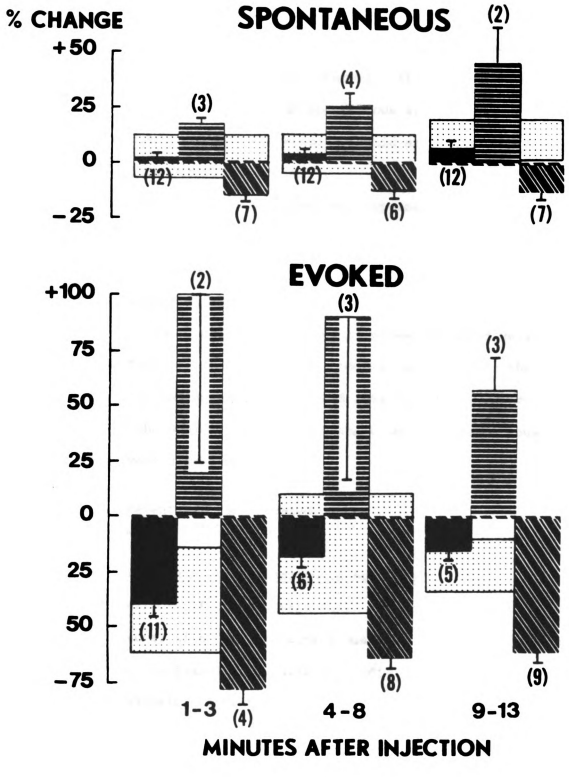
% CHANGE
SPONTANEOUS
+50
25
0
−25
(3)
(12)
(7)
(4)
(12)
(6)
(2)
(12)
(7)
EVOKED
+100
75
50
25
0
25
50
−75
(2)
(11)
(4)
(3)
(6)
(8)
(3)
(5)
(9)
1-3
4-8
9-13
MINUTES AFTER INJECTION

Figure 8

volume was reduced to 1 1. Although a 2 µl volume had minimal effects in the medulla, it may have been too large to inject into the hypothalamus. As shown in Figure 9, these precautions did decrease the size of the confidence interval. It centered more closely around zero when effects on spontaneous activity were measured. However, 1 µl of cerebrospinal fluid still caused a relative inhibition of evoked activity.

Again, ouabain (100 ng) injected into hypothalamic sites frequently had no effect on spontaneous activity of the stellate preganglionic nerve. A few relatively small changes were observed. Considerably greater effects of ouabain were seen on evoked activity. In a number of trials, effects on evoked responses extended well beyond the confidence limits. However, some injections into the hypothalamus also had no effect on evoked activity of this nerve. Inhibition was the dominant effect of ouabain on both spontaneous and evoked nervous discharges.

Effects of Intravenously Administered Digoxin on Sympathetic Activity

Effects of Digoxin on Postganglionic Activity

The effects of digoxin were first observed in cats in which all possible neural sites of drug action could potentially influence nerve activity. Postganglionic inferior cardiac nerve activity was recorded in 6 animals with intact chemoreceptor and baroreceptor reflexes. One example of the effects of increasing doses of digoxin on electrocardiogram, blood pressure and postganglionic nerve activity is

Figure 9. Quantified effects of injections of 100 ng ouabain into hypothalamic cardio-accelerator sites on activity of stellate preganglionic nerve.

Format is the same as described for Figure 4.

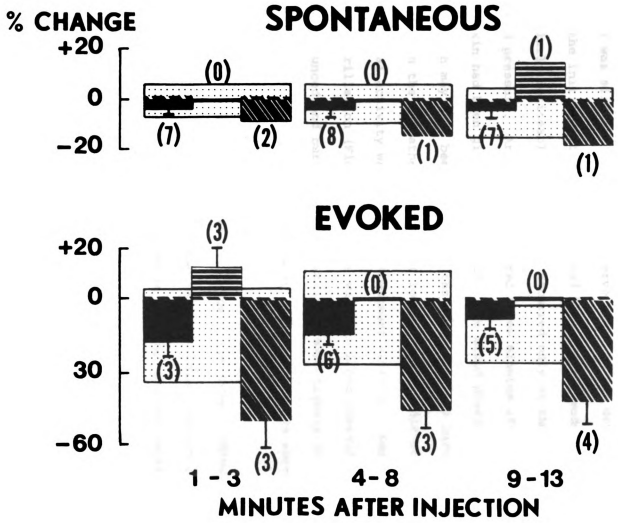
% CHANGE
SPONTANEOUS
+20
0
−20
(0)
(7)
(2)
(0)
(8)
(1)
(1)
(7)
(1)
EVOKED
+20
0
30
−60
(3)
(3)
(3)
(0)
(6)
(3)
(0)
(5)
(4)
1 - 3
4 - 8
9 - 13
MINUTES AFTER INJECTION

Figure 9

shown in Figure 10. The initial pre-drug activity (Figure 10A) had a typical bursting pattern. The 1:1 relationship between bursts and diastole indicate baroreceptor reflex modulation. At a dose of 100 μg/kg (Figure 10B) the electrocardiogram was still normal, the blood pressure was slightly elevated and nerve activity was depressed to 52% of the initial activity. Increased baroreceptor modulation was particularly noticeable since bursts occurred only at the low phases of blood pressure. Arrhythmia occurred a few minutes after 160 μg/kg of digoxin had been given (Figure 10C). The blood pressure contained waves which may have been related to the arrhythmia and bursting activity in the sympathetic nerve was locked to the nadir of each wave. Total activity was 91% of pretreatment activity. Seconds before fibrillation (Figure 10D), the waves in blood pressure were more pronounced and bursts in nerve activity were tightly locked to the nadir of each wave. Total activity was increased 76% above initial pre-drug activity.

However, as shown in Figure 11, digoxin had several effects on nerve activity in these cats. In two cats, digoxin produced progressive increases in activity as great as 2 and 5 times initial activity. In two others, digoxin inhibited activity to 16% and 28% of pre-drug activity. In another cat digoxin had little effect on activity and in the last cat digoxin had a biphasic effect with an initial inhibition to 38% of initial activity followed by almost a two-fold increase above pre-drug activity. Because of the large variability between cats in their responses to digoxin, analysis of variance of these data did not detect effects due to digoxin treatment.

Figure 10. Effects of digoxin in a cat with intact chemoreceptor and baroreceptor afferent nerves.

Drug effects were observed on EKG (top row), blood pressure (middle row) and inferior cardiac nerve activity (bottom row). EKG is illustrated at a faster paper speed than blood pressure or nerve activity. Systolic and diastolic blood pressure are indicated beside each blood pressure record. Control parameters are shown in A. In B, the dose of digoxin was 100 µg/kg. EKG was still relatively normal, blood pressure had increased and nerve activity was depressed to 52% of pre-drug activity. In C, at 160 µg/kg, arrhythmia had occurred. Bursting activity in the nerve was locked to the nadir of each blood pressure wave. Total activity was 91% of pre-drug activity. D shows events occurring seconds before fibrillation. The arrhythmia was severe and total nerve activity was increased 76% above pre-drug activity.

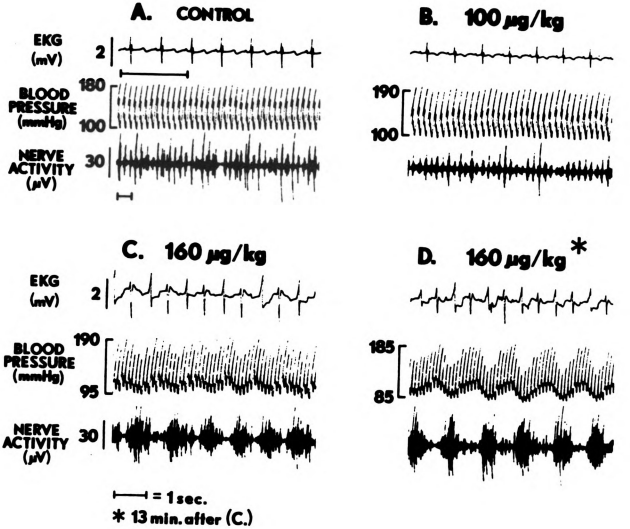
A. CONTROL
B. 100 µg/kg
C. 160 µg/kg
D. 160 µg/kg *
EKG (mV)
2
BLOOD PRESSURE (mmHg)
180
100
190
100
NERVE ACTIVITY (µV)
30
190
95
185
85
= 1 sec.
* 13 min. after (C.)

Figure 10

Figure 11. Effect of cumulative doses of digoxin on postganglionic inferior cardiac nerve activity in 6 cats with intact chemoreceptor and baroreceptor afferent nerves.

The doses of digoxin are plotted on the abscissa and sympathetic activity expressed as a percent of control on a logarithmic scale is plotted on the ordinate. Sympathetic activity was quantified from cumulative integration epoch time intervals with pre-drug time equated to 100%. The mean pre-drug epoch time was 66 $\pm$ 9 sec and the integrator reset at 672 $\pm$ 100 $\mu V \cdot sec$. The dotted line at 100% is a control reference line. Each solid line represents the responses of one cat. The line becomes interrupted at the dose which initiated arrhythmia and the last measurement was made immediately preceding ventricular fibrillation. Digoxin had several effects on postganglionic activity in these cats. It inhibited activity in 3 cats, enhanced activity in 2 cats, had little effect in 1 cat and caused a biphasic response in 1 cat.

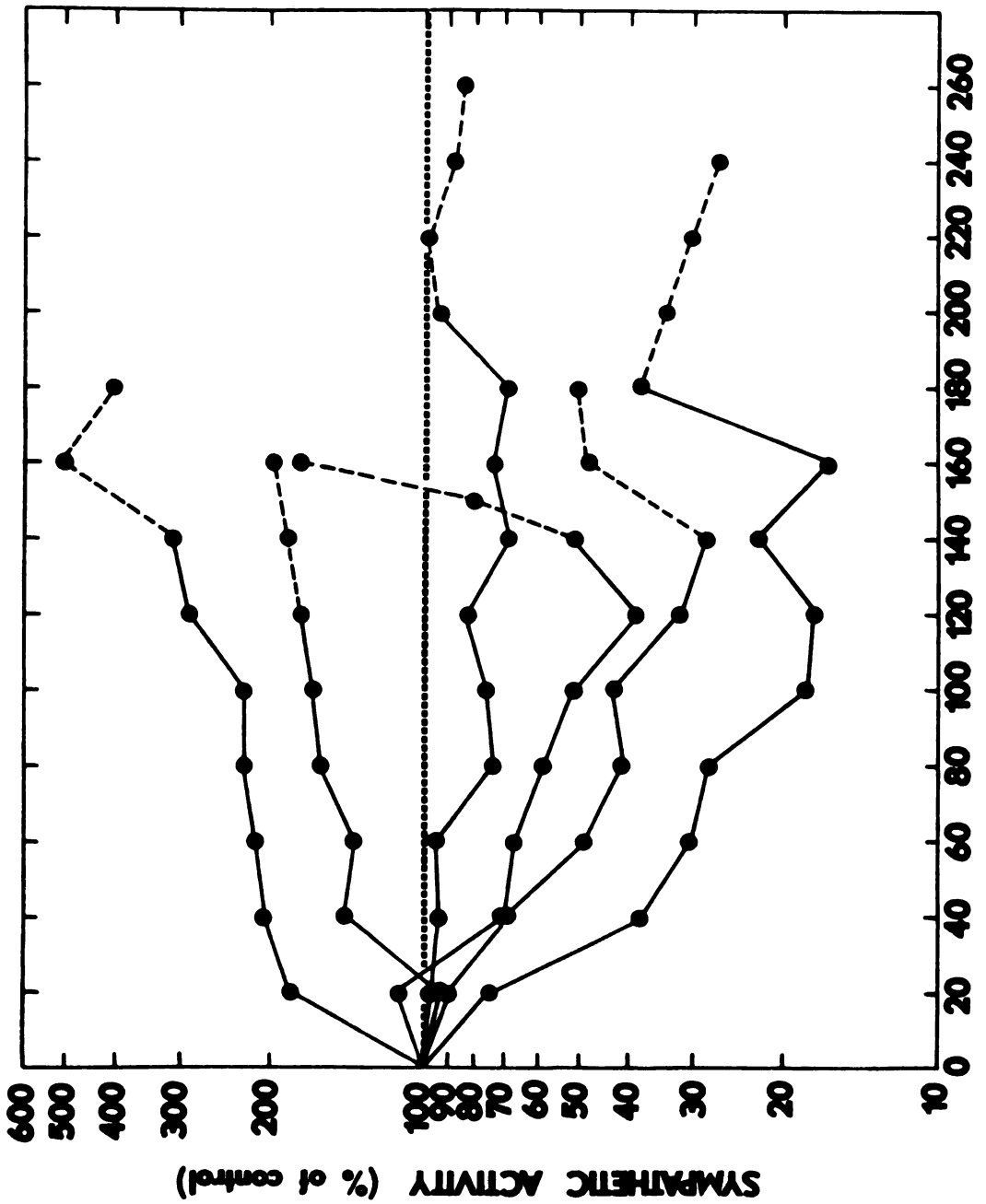
SYMPATHETIC ACTIVITY (% of control)
600
500
400
300
200
100
80
70
60
50
40
30
20
10
0
20
40
60
80
100
120
140
160
180
200
220
240
260
DOSE OF DIGOXIN (µg/kg)

Figure 11

Since differences between animals increased with dose (Figure 11), the only statistical change with dose was an increased variance. There was a tendency for nerve activity to increase after the onset of arrhythmia. This increase was coincident with falling blood pressure in 4 of the 6 cats. The mean dose of digoxin to evoke arrhythmia was 153 $\pm$ 12 µg/kg.

In a second group, effects of digoxin were observed on postganglionic inferior cardiac nerve activity in 6 cats in which the ninth and tenth cranial nerves had been sectioned. In these cats, no changes in postganglionic activity could occur due to the drug effects on chemoreceptor or baroreceptor reflexes. Effects of digoxin in a cat representative of this group are illustrated in Figure 12. At a dose of 80 µg/kg digoxin increased blood pressure and doubled postganglionic nerve activity. Arrhythmia occurred at 100 µg/kg, and blood pressure and sympathetic activity were increased further. Seconds before fibrillation, at 120 µg/kg of digoxin, blood pressure fell, perhaps due to the severity of the arrhythmia, and nerve activity was 5 times the initial activity.

The effect of digoxin on postganglionic activity in all 6 cats in this group is shown in Figure 13. In all of these cats the sole effect of digoxin was to increase sympathetic discharge. Increases as great as 3 and 5 times pre-drug activity occurred. Analysis of variance was performed on nerve responses to digoxin (doses 0-100 µg/kg). Since one cat died at 100 µg/kg, analysis could not be extended to higher doses and still include all of the animals. However, the treatment effect was significant in this dose range. The

Figure 12. Typical effects of digoxin in cat with severed chemoreceptor and baroreceptor afferent nerves.

Drug effects were observed on EKG (top row), blood pressure (middle row) and postganglionic inferior cardiac nerve activity (bottom row). The numbers labeled % of control are the quantitations of the nerve activity illustrated directly above the number. Digoxin increased blood pressure and nerve activity at doses of 80 and 100 µg/kg. Arrhythmia occurred at 100 µg/kg. Immediately before fibrillation, at 120 µg/kg, the arrhythmia was severe, blood pressure had fallen and nerve activity was greatly enhanced.

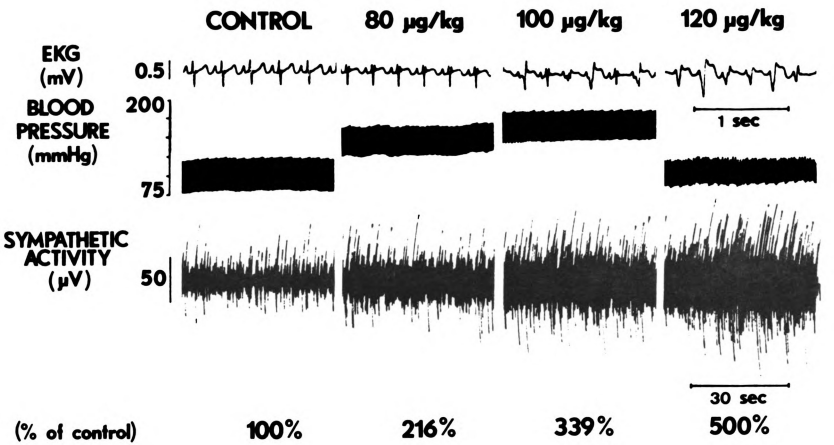
CONTROL
80 µg/kg
100 µg/kg
120 µg/kg
EKG
(mV)
0.5
BLOOD
PRESSURE
(mmHg)
200
75
1 sec
SYMPATHETIC
ACTIVITY
(µV)
50
30 sec
(% of control)
100%
216%
339%
500%

Figure 12

Figure 13. Effects of cumulative doses of digoxin on postganglionic inferior cardiac nerve activity in 6 cats with severed chemoreceptor and baroreceptor afferent nerves.

The format is the same as that of Figure 11. The mean pre-drug epoch time was 82 ± 8 sec and the integrator reset at 992 ± 99 μV·sec. The sole effect of digoxin in all 6 cats in this group was to increase activity.

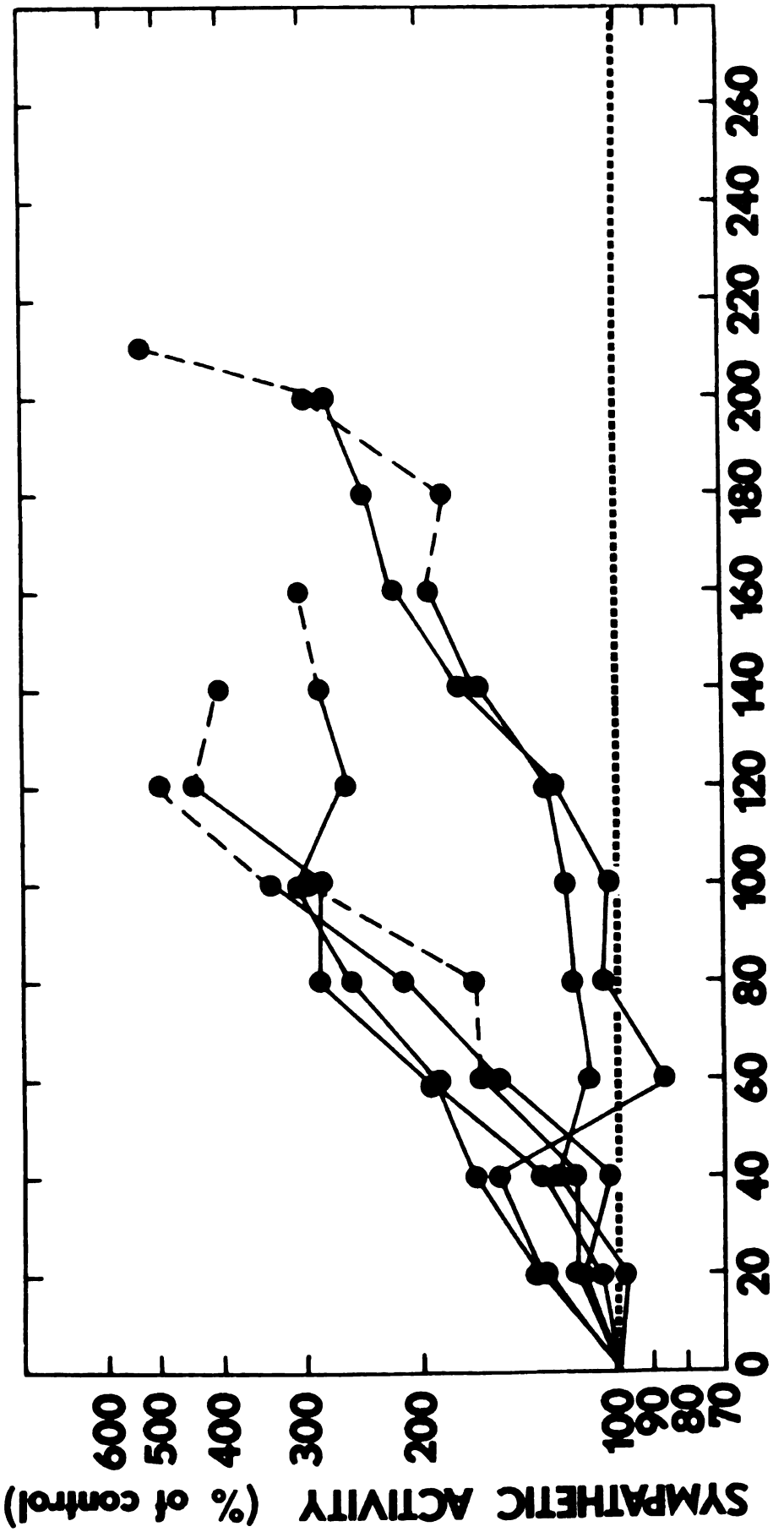
SYMPATHETIC ACTIVITY (% of control)
600
500
400
300
200
100
90
80
70
0
20
40
60
80
100
120
140
160
180
200
220
240
260
DOSE OF DIGOXIN (µg/kg)

Figure 13

mean responses at 80 µg/kg and 100 µg/kg were significantly different from pre-drug activity. Since it is apparent from Figure 3 that after 100 µg/kg the increases continued to get larger, digoxin clearly increased postganglionic nerve activity in these cats. The mean dose to evoke arrhythmia was 130 $\pm$ 20 µg/kg in this group.

Effects of Digoxin on Preganglionic Activity

Effects of digoxin were next observed on preganglionic splanchnic nerve activity. In these cats, drug effects in the ganglion could not contribute to the changes in preganglionic sympathetic nerve activity. Digoxin was first administered to 6 cats with intact baroreceptor and chemoreceptor reflexes. Figure 14 illustrates the effects of digoxin on EKG, blood pressure and splanchnic nerve activity in a cat representative of this group. Baroreceptor reflex modulation of nerve activity was apparent in this cat, as shown in the control panel of Figure 14. Activity was inhibited at the peaks in blood pressure and bursting occurred at the lower pressures. Blood pressure was increased slightly by 100 µg/kg digoxin and preganglionic activity was inhibited to 36% of pre-drug activity. The bursts in activity were smaller and the periods of inhibition were longer compared to initial activity. At 140 µg/kg, cardiac arrhythmia occurred, blood pressure approximately equalled pre-drug levels and nerve activity was still reduced to 46% of initial activity. Seconds before fibrillation (which occurred 12 min after the last dose of digoxin) the arrhythmia was severe, blood pressure had fallen and nerve activity had increased, but it was still less than initial activity (Figure 14, last panel).

Figure 14. Typical effects of digoxin in a cat with intact chemoreceptor and baroreceptor afferent nerves.

Sympathetic activity was recorded from the preganglionic splanchnic nerve in this cat. In all other respects, the format is the same as that of Figure 12. Digoxin markedly inhibited preganglionic activity at doses of 100 and 140 µg/kg. Blood pressure was increased slightly at 100 µg/kg and arrhythmia occurred at 140 µg/kg. The last panel shows responses immediately preceding fibrillation which occurred 12 min after the last injection of digoxin. The arrhythmia was severe, blood pressure was slightly less than pre-drug levels and nerve activity was still less than pre-drug activity.

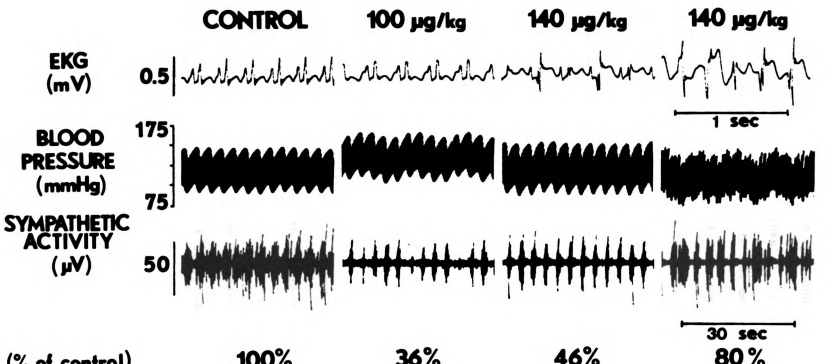
CONTROL
100 µg/kg
140 µg/kg
140 µg/kg
EKG
(mV)
0.5
1 sec
BLOOD
PRESSURE
(mmHg)
175
75
SYMPATHETIC
ACTIVITY
(µV)
50
30 sec
(% of control)
100%
36%
46%
80%

Figure 14

The effect of digoxin on preganglionic splanchnic activity in all 6 cats in this group is illustrated in Figure 15. Digoxin decreased activity in all of these cats. Inhibition to 36% and 42% of pre-drug activity occurred. Analysis of variance of the responses to digoxin (0-140 µg/kg) showed significant treatment effects. The mean nerve activity responses at each dose from 40 to 140 µg/kg were significantly different from pre-drug activity. Since 2 cats died at 140 µg/kg, the statistical analysis could not be extended beyond this dose. There was a tendency for nerve activity to return back toward pre-drug levels in each cat as toxicity progressed toward an arrhythmia. However, at the same time, blood pressure had begun to fall, perhaps due to the toxic drug effects on the heart.

The relationship between mean blood pressure and preganglionic nerve activity in the same 6 cats is illustrated in Figure 16. The mean nerve activity and blood pressure responses for all 6 animals were compared at increasing doses of digoxin. When the last pre-fibrillatory response had to be measured at a dose between the 20 µg increments plotted on Figure 16, it was included in the higher dose group. Digoxin (100 µg/kg) increased blood pressure approximately 10 mmHg and inhibited nerve activity to 60% of pre-drug activity. Starting at approximately 120 µg/kg, nerve activity returned back toward pre-drug levels associated with decreasing blood pressure. At the mean arrhythmic dose (158 $\pm$ 8 µg/kg), blood pressure had fallen 20 mmHg and nerve activity had increased to 83% of pre-drug activity.

Figure 15. Effects of cumulative doses of digoxin on preganglionic splanchnic nerve activity in 6 cats with intact chemoreceptor and baroreceptor reflexes.

The format is the same as that of Figure 11. The mean pre-drug epoch time was 81 ± 12 sec and the integrator reset at 150 ± 46 μV·sec. Digoxin inhibited activity in all 6 cats in this group. At approximately the arrhythmic dose of digoxin, activity tended to increase back toward pre-drug activity.

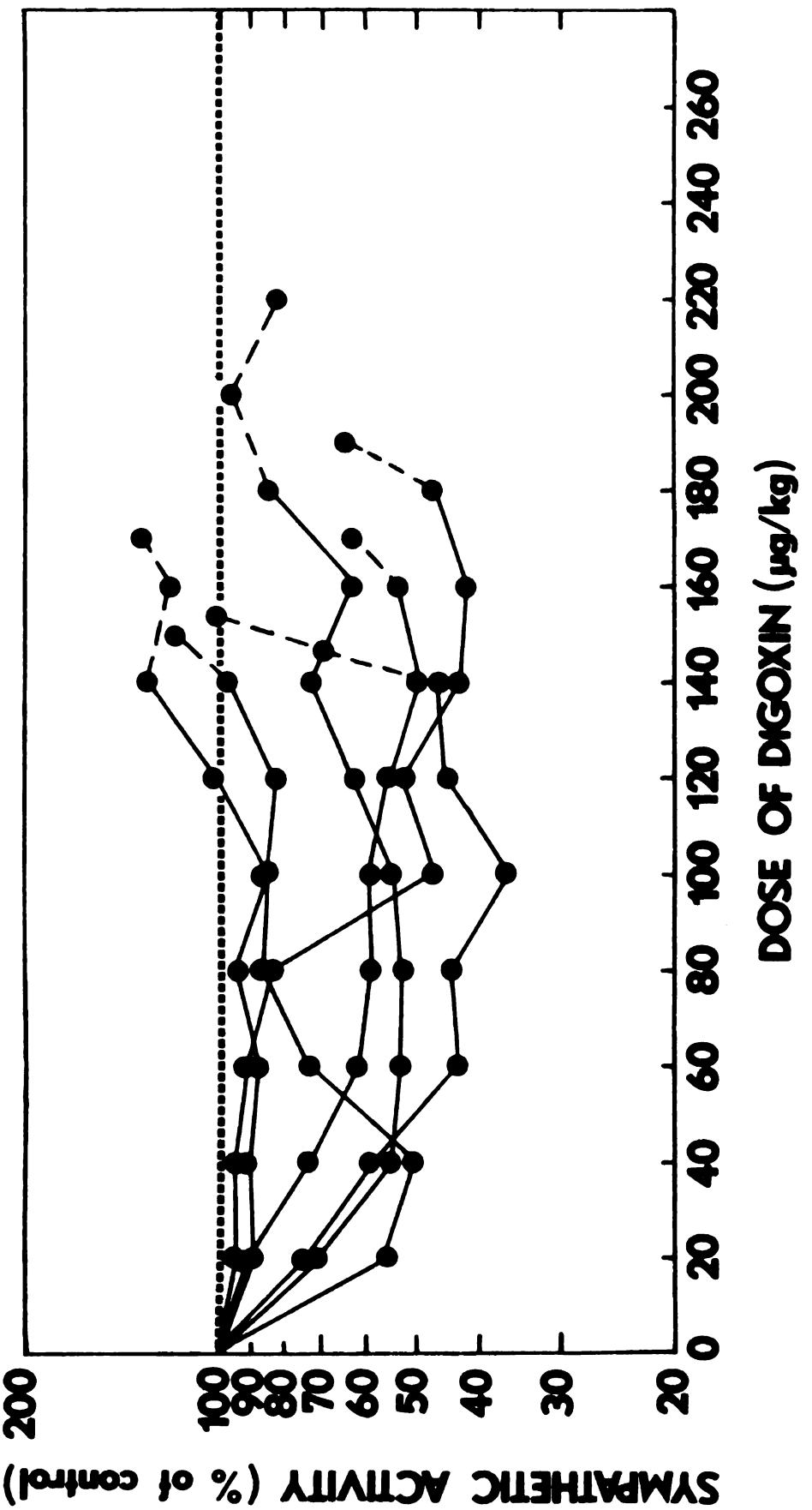
SYMPATHETIC ACTIVITY (% of control)
200
100
90
80
70
60
50
40
30
20
0
20
40
60
80
100
120
140
160
180
200
220
240
260
DOSE OF DIGOXIN (µg/kg)

Figure 15

Figure 16. Comparison of effects of cumulative doses of digoxin on blood pressure and preganglionic splanchnic nerve activity in 6 cats with intact chemoreceptor and baroreceptor reflexes.

The doses of digoxin are plotted on the abscissa. Mean blood pressure (mmHg) is plotted on the left vertical axis and sympathetic activity (% of pre-drug activity) is plotted on the right vertical axis. The responses of the 6 cats at each dose are expressed as a mean $\pm$ SEM. Blood pressure is indicated by closed circles, nerve activity by open circles and the mean arrhythmic dose by an asterisk. Mean blood pressure was increased and nerve activity inhibited by 40-100 µg/kg digoxin. Blood pressure then began to fall at higher doses, and nerve activity increased back toward pre-drug levels of activity.

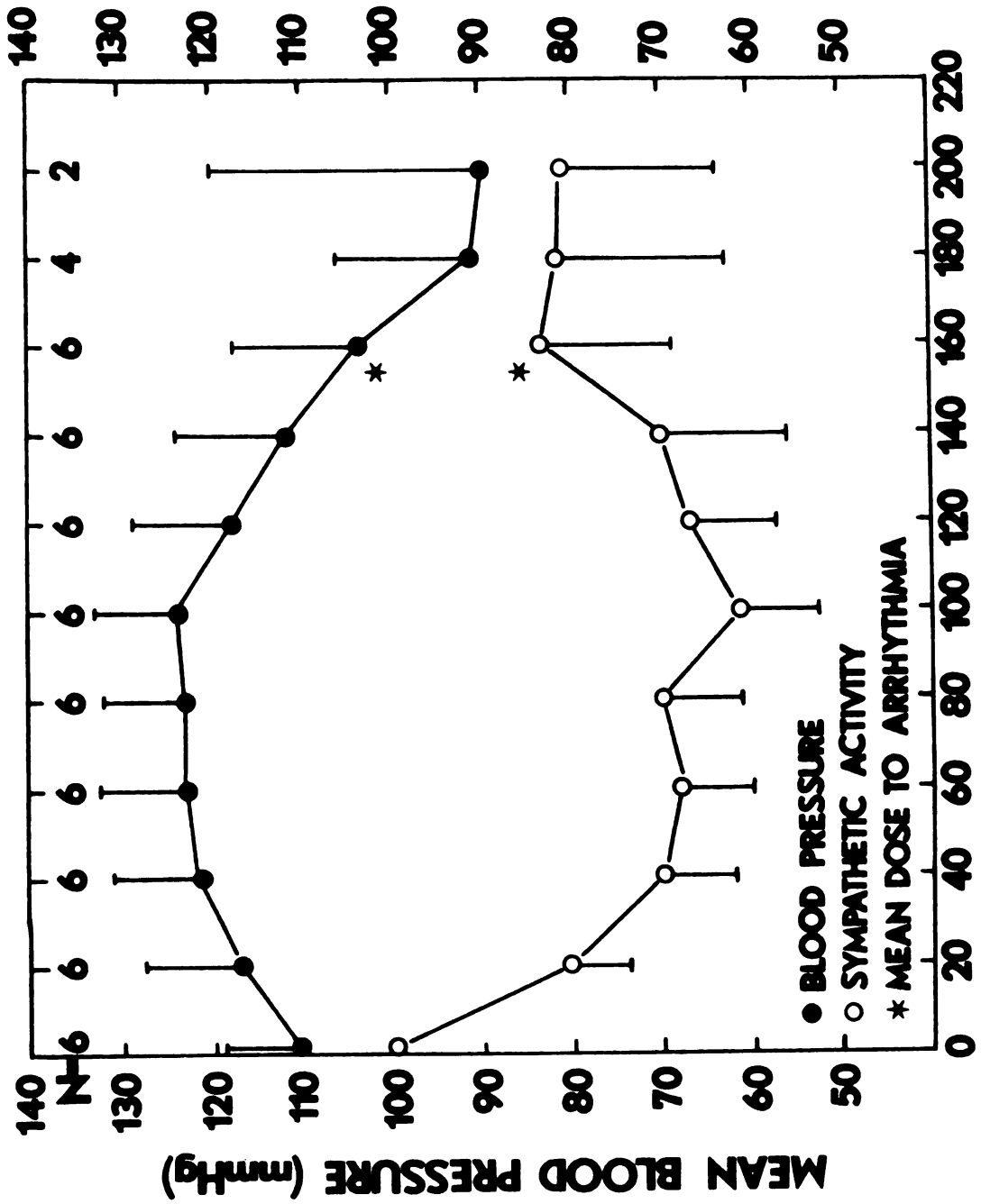
MEAN BLOOD PRESSURE (mmHg)
SYMPATHETIC ACTIVITY (% of control)
DOSE OF DIGOXIN
N
6 6 6 6 6 6 6 6 6 4 2
● BLOOD PRESSURE
○ SYMPATHETIC ACTIVITY
★ MEAN DOSE TO ARRHYTHMIA
140 130 120 110 100 90 80 70 60 50
0 20 40 60 80 100 120 140 160 180 200 220

Figure 16

In the last group, effects of digoxin were observed on splanchnic preganglionic activity in cats with severed ninth and tenth cranial nerves. Thus, chemoreceptor and baroreceptor as well as ganglionic effects of digoxin could not alter the observed sympathetic activity. Digoxin produced very little change in nerve activity in these 7 cats (Figure 17). In 3 cats, preganglionic activity did not vary from initial activity. In 4 others, activity changed only slightly. Analysis of variance showed no treatment effect for the doses 0-100 µg/kg and none of the mean responses in this dose range were significantly different from pre-drug activity. The mean arrhythmic dose of digoxin was 80 $\pm$ 8.7 µg/kg in this group.

Effects of Intravenously Administered Digoxin on Phrenic Nerve Activity

Control Phrenic Nerve Activity

Preliminary experiments were performed in which phrenic nerve activity was recorded for 30-180 min in cats with severed ninth and tenth cranial nerves. Blood-gases, pH and expired CO_2 were not monitored in these cats. These experiments were conducted to evaluate the stability of phrenic recordings during time intervals needed to administer toxic doses of digoxin. As shown in the left column of Table 2, phrenic activity was not constant with respect to time. The large standard errors around mean activity at each time interval of measurement illustrate the large variability of nerve activity. In some cats nerve activity gradually increased with time; in others it gradually decreased. This phenomenon appeared to relate to the initial rate or volume of

Figure 17. Effects of cumulative doses of digoxin on preganglionic splanchnic nerve activity in 7 cats with severed chemoreceptor and baroreceptor afferent nerves.

The format is the same as that of Figure 11. The mean pre-drug epoch time was 62 ± 4 sec and the integrator reset at 261 ± 51 μV·sec. Digoxin had no significant effect on nerve activity in these 7 cats. In 3 cats activity did not vary from pre-drug levels and in 4 others it changed only slightly.

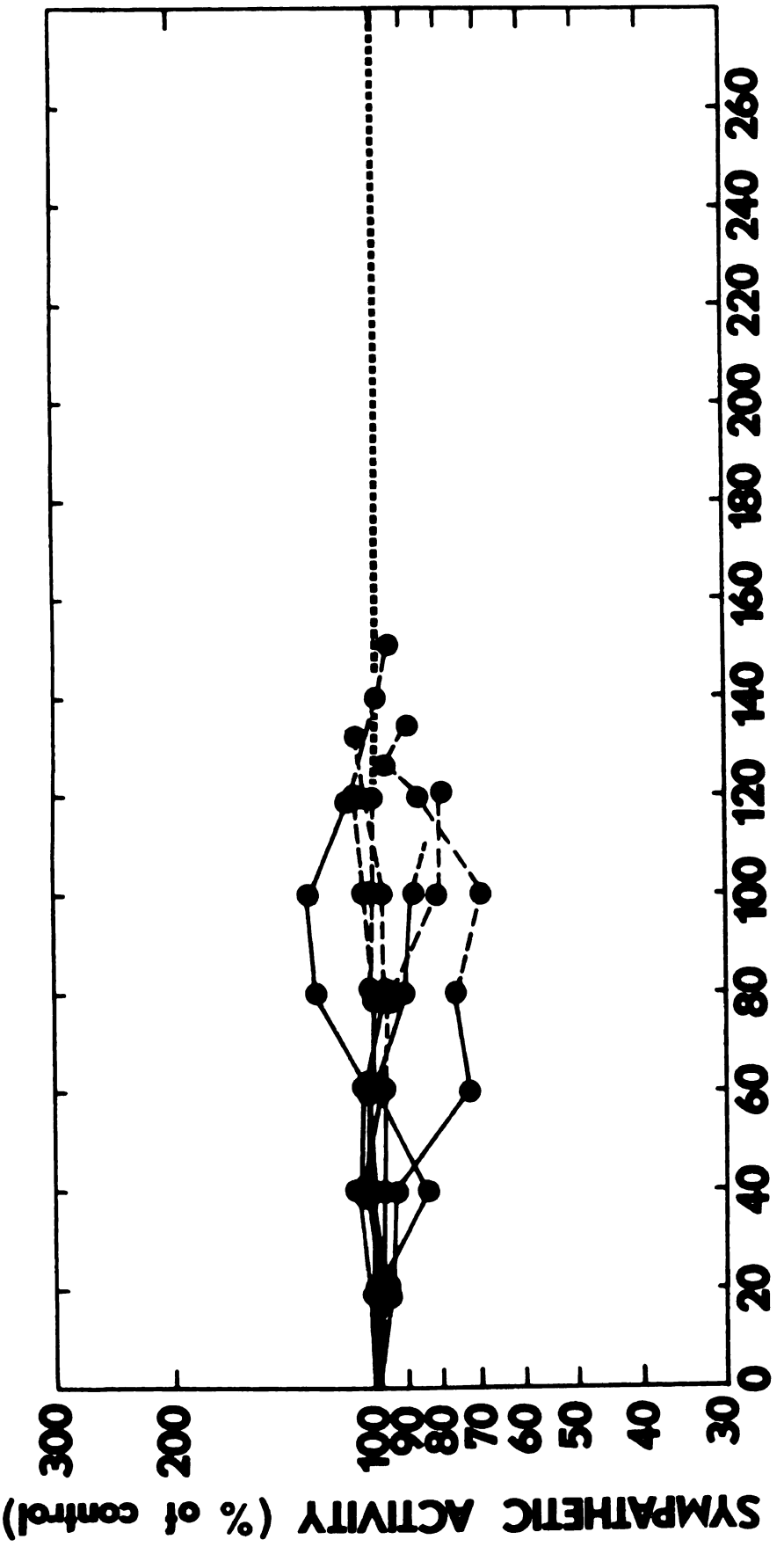
SYMPATHETIC ACTIVITY (% of control)
300
200
100
80
70
60
50
40
30
0
20
40
60
80
100
120
140
160
180
200
220
240
260
DOSE OF DIGOXIN (µg/kg)

Figure 17

Table 2. Control phrenic nerve activity without CO_2 monitoring compared with activity from cats in which end-tidal CO_2 was monitored and stabilized

Time[a]	A: End-tidal CO_2 unadjusted		B: End-tidal CO_2 stabilized	
	Phrenic activity[b]	N[c]	Phrenic activity	N
0	100	9	100	9
15	109 ± 10.0	9	102.0 ± 6.8	9
30	124.1 ± 15.6	9	116.2 ± 7.7	9
45	136.8 ± 46.2	7	109.2 ± 6.7	9
60	134.0 ± 31.1	7	100.2 ± 6.9	6
120	95.1 ± 49.8	5		
180	122.3 ± 34.0	4		

[a]Elapsed time in min.

[b]Phrenic nerve activity expressed as a mean percent of initial activity ± SEM. The initial epoch time for Group A was 76 ± 10 sec and the integrator reset at 239 ± 29 μV·sec. The initial epoch time for Group B was 71 ± 7 sec and the integrator reset at 186 ± 61 μV·sec.

[c]Number of cats observed at each time interval.

respiration since the changing activity could be stabilized with very subtle alterations in respiratory rate (.2-.5 RPM). This suggested that although the cats appeared to be respired adequately, they were sometimes slightly hypoventilated and sometimes slightly hyperventilated. This could lead to alterations in arterial pH, pCO_2 and pO_2, all of which influence respiratory motor activity. It was apparent that drug effects could not be clearly identified on such an unstable nerve preparation.

In a second series of control experiments, pH, pCO_2, pO_2 (the physiological stimulants to respiration) and end-tidal CO_2 were monitored and maintained as constant as possible in an attempt to stabilize phrenic nerve activity. This was accomplished by correction of acidosis with infusions of sodium bicarbonate and by subtle adjustments of the respiratory rate to keep end-tidal CO_2 constant. The mean control phrenic nerve activity in these cats is shown in the right column of Table 2. While the mean activity changed slightly, the variability was greatly reduced when arterial pH, blood gases and end-tidal CO_2 were monitored and maintained constant.

Effects of Digoxin on Phrenic Nerve Activity in Cats with Intact IX and X Cranial Nerves

Effects of accumulating doses of digoxin on spontaneous phrenic activity were observed in 6 cats. In these cats end-tidal CO_2 was maintained constant throughout the experiment (Table 3, left column).

An example of cardiac and phrenic nerve activity responses to digoxin are shown in Figure 18. A subarrhythmic dose of digoxin (140 µg/kg) increased the amplitude and duration of each burst of

Table 3. End-tidal CO_2 during administration of digoxin in cats with intact or severed IX and X cranial nerves

Digoxin (µg/kg)	Denervated[a] End-tidal CO_2 (mmHg)	N[c]	Intact[b] End-tidal CO_2 (mmHg)	N
0	32.2 ± 2.5[d]	5	41.9 ± 3.0[d]	8
20	32.4 ± 2.5	5	42.2 ± 3.0	8
40	32.5 ± 2.5	5	42.7 ± 3.5	8
60	33.9 ± 2.8	5	42.1 ± 3.4	8
80	33.5 ± 2.9	5	42.0 ± 3.1	8
100	34.0 ± 2.6	4	38.6 ± 3.2	7
120	33.6 ± 3.0	4	37.9 ± 3.3	7
140	33.4 ± 3.1	4		
160	33.3 ± 3.1	4		
180	34.6 ± 4.1	3		

[a] Cats with severed IX and X cranial nerves.

[b] Cats with intact IX and X cranial nerves.

[c] Number of cats whose end-tidal CO_2 was measured at this dose.

[d] Data are expressed as means ± SEM.

Figure 18. Effects of digoxin on phrenic nerve activity and EKG in a cat with intact IX and X cranial nerves.

The upper row is EKG, the second row is phrenic nerve activity recorded at a fast paper speed, the third row is the same phrenic nerve activity recorded at a slower paper speed and the bottom row is the cumulative integration of phrenic nerve activity. Digoxin (140 µg/kg) doubled phrenic nerve activity and shortened the integration epoch time. Arrhythmia occurred at 180 µg/kg and ventricular fibrillation occurred seconds after the record illustrated here (180 µg/kg). Phrenic nerve activity at 180 µg/kg was increased to 317% of pre-drug activity.

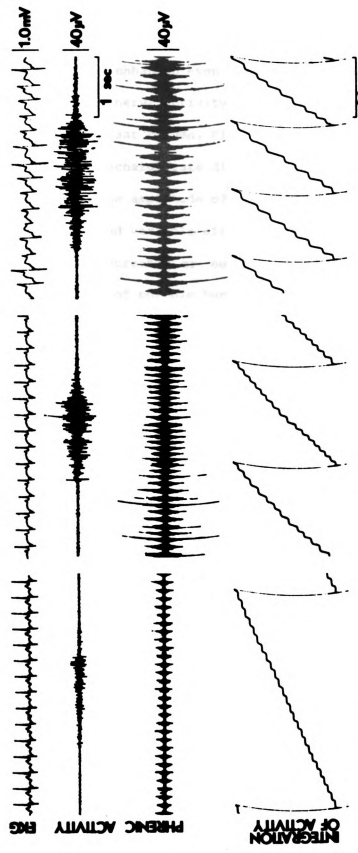
1.0mV
40μV
1 sec
40μV
30 sec
ERG
ACTIVITY
PHRENIC
INTEGRATION OF ACTIVITY
CONTROL
140 μg/kg
180 μg/kg

Figure 18

activity. Total integrated activity was doubled by 140 µg/kg. Phrenic nerve activity was enhanced even further by the arrhythmic dose (180 µg/kg). Phrenic nerve activity occurring seconds before ventricular fibrillation (last column, Figure 18) was 317% of pre-drug activity. Phrenic discharge rate did not change in this cat. Digoxin always increased the amplitude of activity in each phrenic burst in these cats. It had variable effects on the duration of each burst causing increases, decreases or no change in burst duration. Digoxin increased the rate of phrenic bursting in 4 of the 6 cats.

The effect of digoxin on phrenic nerve activity in all 6 cats in this group is shown in Figure 19. Digoxin increased activity in all of these cats. Activity began to increase at 100 µg/kg and mean activity was statistically different from pre-drug activity at 120-180 µg/kg. The mean phrenic discharge rate before fibrillation (35 $\pm$ 7 bursts/min) was significantly greater than the initial rate (15 $\pm$ 2 bursts/min). The arrhythmic dose of digoxin was approximately 137 µg/kg.

In three of these cats, the ninth and tenth cranial nerves were tightly tied at the jugular foramen after digoxin had evoked arrhythmia and large increases in phrenic nerve activity. This procedure decreased phrenic nerve activity 66%, 69% and 73%. After tying the nerves, activity fell to levels within the pre-drug range of activity. The ninth and tenth cranial nerves were tied in two untreated cats resulting in 16% and 17% decreases in phrenic nerve activity.

Figure 19. Effect of cumulative doses of digoxin on phrenic nerve activity in 6 cats with intact IX and X cranial nerves.

The doses of digoxin are plotted on the abscissa and phrenic nerve activity in a logarithmic scale is plotted on the ordinate. Phrenic nerve activity was quantified from the cumulative integration epoch time intervals with pre-drug time equated to 100%. The mean pre-drug epoch time was 88 $\pm$ 15 sec and the integrator reset at 320 $\pm$ 98 $\mu V \cdot sec$. The dotted line at 100% is a control reference line. The response to each dose represents the mean nerve activity $\pm$ SEM. The number of cats are indicated in parentheses. The number of cats observed became smaller at higher doses as the cats died. Digoxin increased phrenic nerve activity in these 6 cats with significant mean increases from pre-drug activity at 120-180 $\mu g/kg$.

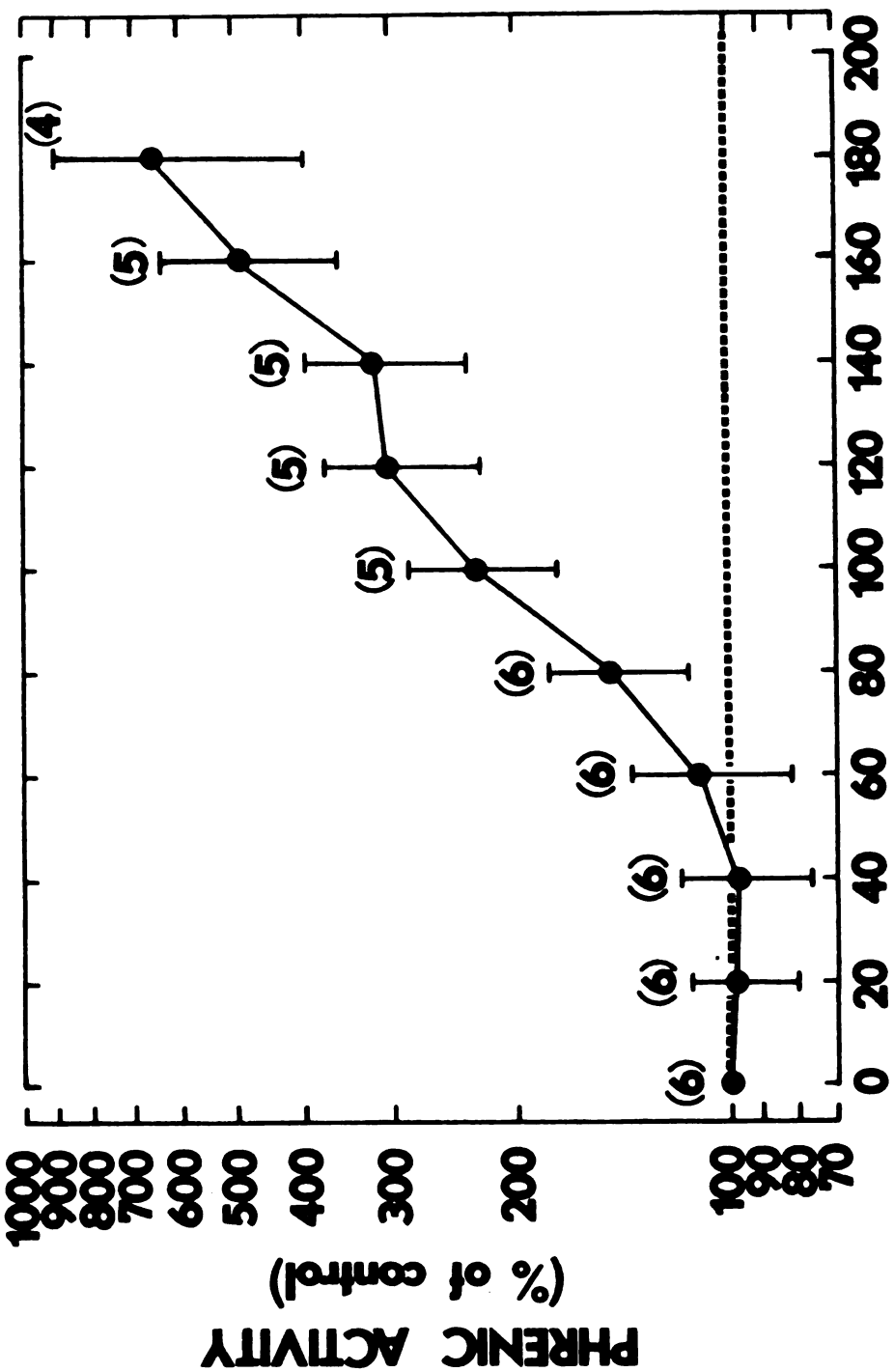
PHRENIC ACTIVITY
(% of control)
1000
900
800
700
600
500
400
300
200
100
90
80
(4)
(5)
(5)
(5)
(5)
(6)
(6)
(6)
(6)
(6)
0
20
40
60
80
100
120
140
160
180
200
DOSE OF DIGOXIN (µg/kg)

Figure 19

Effects of Digoxin on Phrenic Nerve Activity in Cats with Severed IX and X Cranial Nerves

The effects of digoxin were observed in 9 cats whose afferent chemoreceptor and pressoreceptor influence on phrenic nerve activity had been eliminated by sectioning the ninth and tenth cranial nerves. Afferent impulses from stretch receptors in intercostal muscles were minimized by a pneumothoracotomy which eliminated chest movement with respiration. End-tidal CO_2 was kept constant in these cats (Table 3, right column). Typical responses to digoxin from one of these cats are shown in Figure 20. Although digoxin had its usual effect on the heart, the subarrhythmic dose (80 µg/kg) and the arrhythmic dose (120 µg/kg) had no apparent effect on phrenic discharge. Discharge rate and integrated nerve activity were constant throughout the experiment. Small increases in the amplitude, but not rate, of phrenic discharge occurred in 2 of the 9 cats in this group. In another cat the rate of discharge increased with no accompanying change in amplitude. The effect of digoxin on phrenic nerve activity in all 9 cats is shown in Figure 21. Digoxin caused no significant effect in mean activity at any dose. Analysis of variance of all the nerve activity responses to each dose showed no treatment effect. The rate of phrenic bursting before ventricular fibrillation (22.5 $\pm$ 3.2 bursts/min) was not different from the pre-drug rate (19.1 $\pm$ 2.2 bursts/min). The mean arrhythmic dose in these cats was approximately 104 µg/kg.

Figure 20. Effects of digoxin on phrenic nerve activity and EKG in a cat with severed IX and X cranial nerves.

The format is the same as that in Figure 18. Digoxin had no effect on phrenic nerve activity at the subarrhythmic dose (80 µg/kg) or at the arrhythmic dose (120 µg/kg). The integration epoch time was unchanged throughout this experiment.

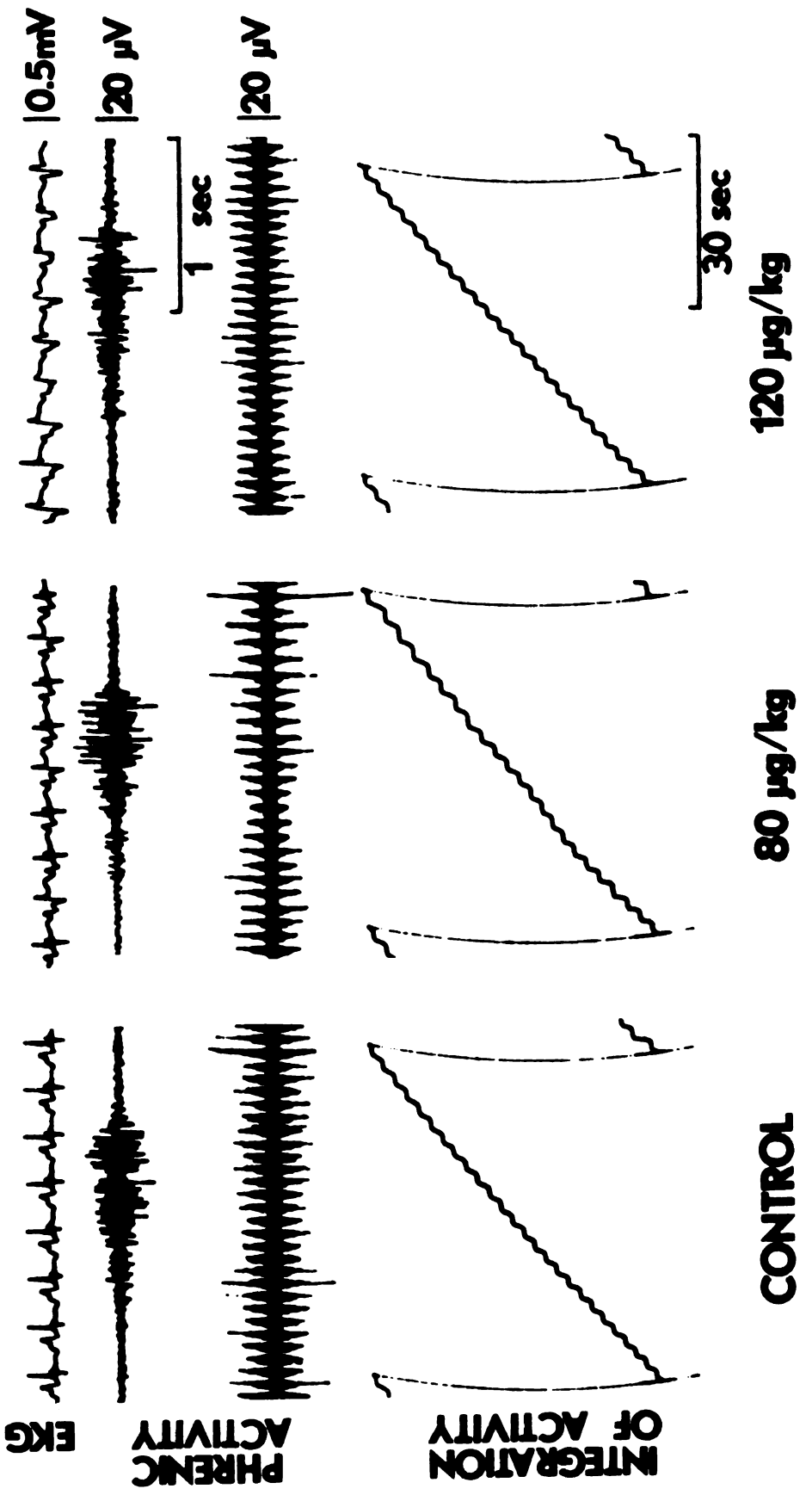
EKG
PHRENIC ACTIVITY
INTEGRATION OF ACTIVITY
0.5mV
20 µV
1 sec
20 µV
30 sec
CONTROL
80 µg/kg
120 µg/kg

Figure 20

Figure 21. Effects of cumulative doses of digoxin on phrenic nerve activity in 9 cats with severed IX and X cranial nerves.

The format is the same as that in Figure 19. Mean pre-drug epoch time was 67 ± 6 sec and the integrator reset at 186 ± 61 μV·sec. Digoxin had no significant effect on nerve activity in these 9 cats at any dose.

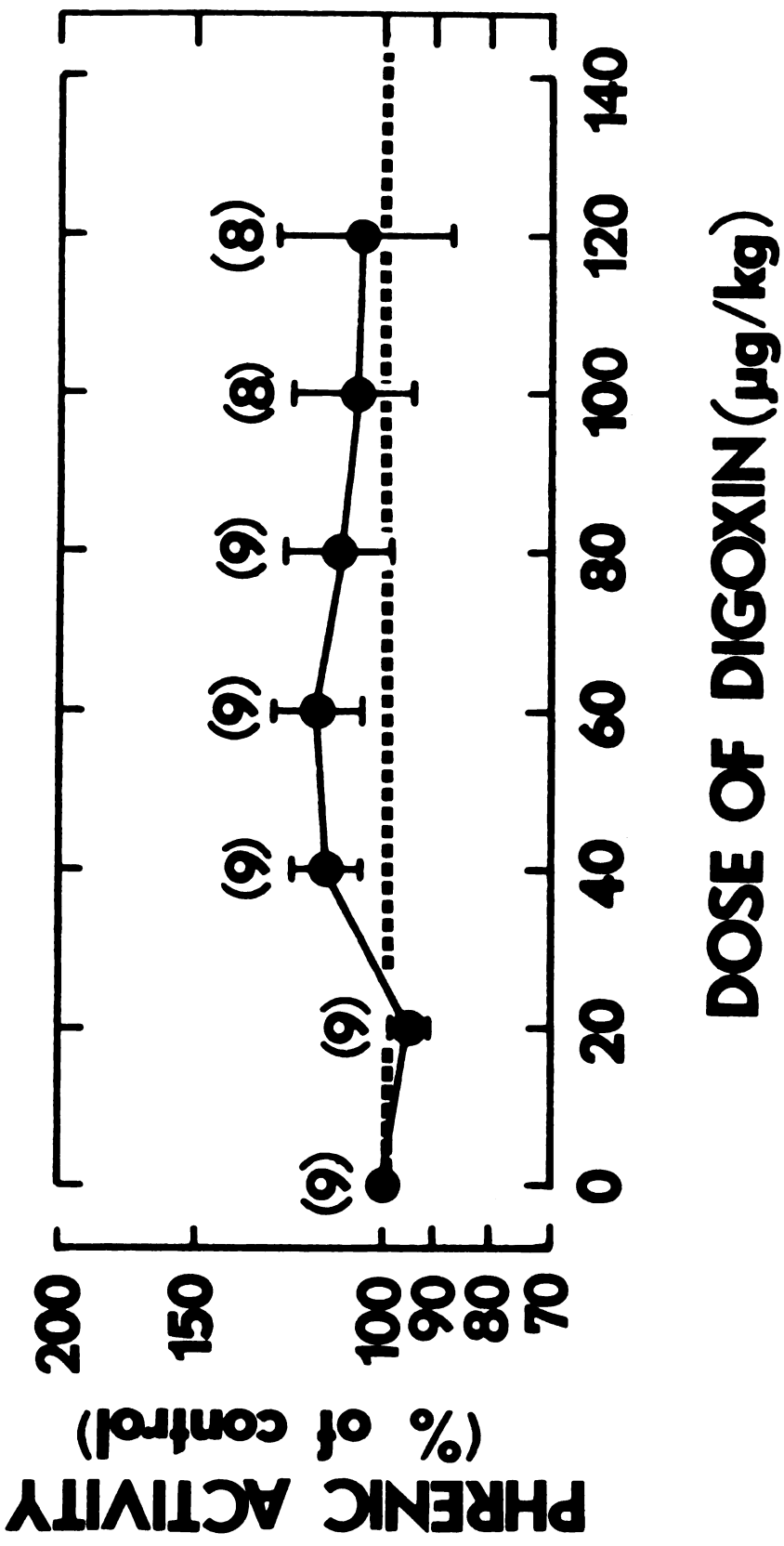
PHRENIC ACTIVITY
(% of control)
200
150
100
90
80
70
(9)
(9)
(9)
(9)
(9)
(8)
(8)
0
20
40
60
80
100
120
140
DOSE OF DIGOXIN (μg/kg)

Figure 21

Concentrations of Digitalis in the Central Nervous System Following Intravenous Administration and Their Effects on Na^+-K^+-ATPase

Concentrations of (^{3}H)-Digoxin in Serum and CSF

Tritium-labeled digoxin (20 μg/kg) was administered intravenously every 15 min to cats anesthetized with the dial-urethan mixture. The concentrations of total serum digoxin, free serum digoxin and that of digoxin in cerebrospinal fluid were determined. The concentrations of digoxin increased with time as the cumulative dose of digoxin was increased (Figure 22). At the mean arrhythmic dose of digoxin (140 $\pm$ 6.5 μg/kg), CSF contained approximately 2 x 10^{-8} M digoxin, whereas free serum digoxin concentrations were about 3.4 x 10^{-8} M and total serum digoxin was approximately 15 x 10^{-8} M. The CSF concentration at death due to ventricular fibrillation was 2.3 x 10^{-8} M.

Randomly labeled drug was used in the first 7 cats. In 2 of these experiments several CSF samples were evaporated to determine if part of their radioactivity was associated with water rather than digoxin. Approximately 70% of the radioactivity in these samples was associated with water. Specifically labeled (12α-^{3}H)-digoxin was used in 2 more cats. In these cats the concentrations of drug appearing in serum samples and CSF samples were quite similar to those found in the earlier experiments. When samples of CSF and serum filtrate containing free digoxin were evaporated, reconstituted and counted, the radioactivity present was within 10% of that

Figure 22. Concentrations of digoxin in CSF and serum in response to cumulative doses of digoxin administered intravenously.

The data are expressed as means ± SEM. The numbers in parentheses above or below each standard error are the number of cats measured at each dose. The serum concentrations have been expressed as total serum digoxin and unbound (free) serum digoxin. The arrow indicates the mean arrhythmic dose for all 9 cats.

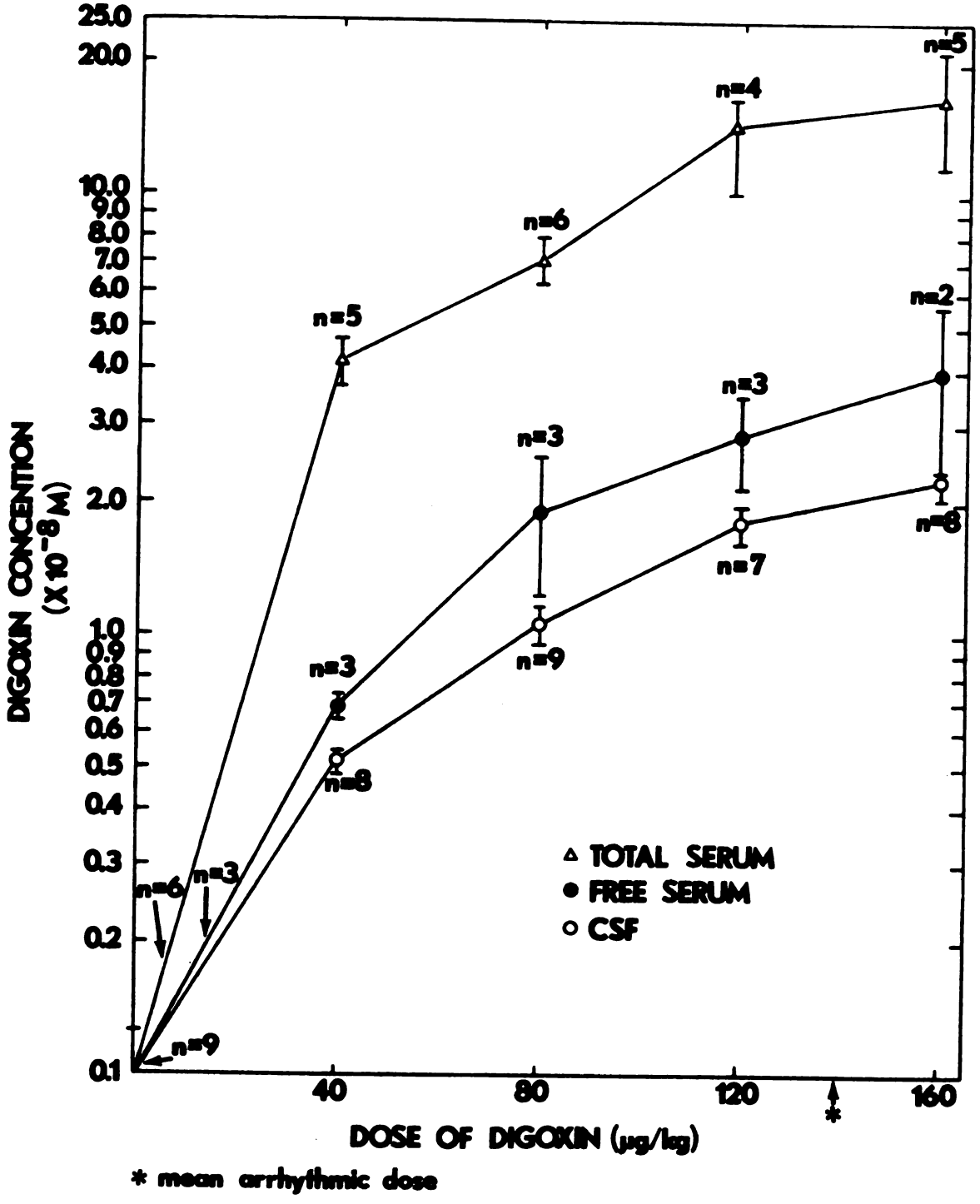
DIGOXIN CONCENTION (X10⁻⁹M)
25.0
20.0
10.0
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
n=6
n=3
n=9
n=5
n=3
n=8
n=6
n=3
n=9
n=4
n=3
n=7
n=5
n=2
n=6
△ TOTAL SERUM
● FREE SERUM
○ CSF
40
80
120
160
*
DOSE OF DIGOXIN (µg/kg)
* mean arrhythmic dose

Figure 22

in unevaporated identical samples. Therefore, it was assumed that the radioactivity in the last 2 cats was associated with digoxin.

Inhibition of Brain Na^+-K^+-ATPase by Digoxin *In Vitro*

Activity of Na^+-K^+-ATPase of the brain particulate fraction was assayed *in vitro* in the presence of various concentrations of digoxin. Activity of Na^+-K^+-ATPase in each of 8 brain areas prior to treatment with digoxin is shown in Figure 23. Activity of Na^+-K^+-ATPase ranged from 21 $\pm$ 8 to 33 $\pm$ 3 μmoles Pi/mg protein/hr. Lower activity was observed in the posterior medulla and the preoptic area. The highest activity was observed in the thalamus and midbrain.

Inhibition of Na^+-K^+-ATPase activity increased with increasing concentrations of digoxin. The lowest concentration of digoxin tested (3×10^{-9} M) had no consistent effect on Na^+-K^+-ATPase (Table 4, Figure 24). A higher concentration of digoxin (1×10^{-8} M) produced a slight inhibition of the enzyme. The average inhibition in the 8 areas was approximately 10%. At 3×10^{-8} M, digoxin inhibited Na^+-K^+-ATPase approximately 29%. Thus, the concentration present in the brain at arrhythmia (2×10^{-8} M) appeared to cause a 10-20% inhibition of the enzyme (Figure 24). The midbrain and pyriform area appeared to be slightly less sensitive to lower concentrations of digoxin than the other brain areas. These apparent regional differences in sensitivity of Na^+-K^+-ATPase to digoxin were not related to regional differences in the enzyme activity. For example, Na^+-K^+-ATPase activity of the midbrain and pyriform area were not different from other areas.

Figure 23. Pre-drug Na^{+}-K^{+}- and Mg^{++}-ATPase activity from cat brain.

The ATPase activity for each of the 8 brain areas before *in vitro* inhibition by digoxin (Table 4, Figure 24) is shown here. The data are expressed as means ± SEM. The numbers in parentheses represent the number of cat brains assayed.

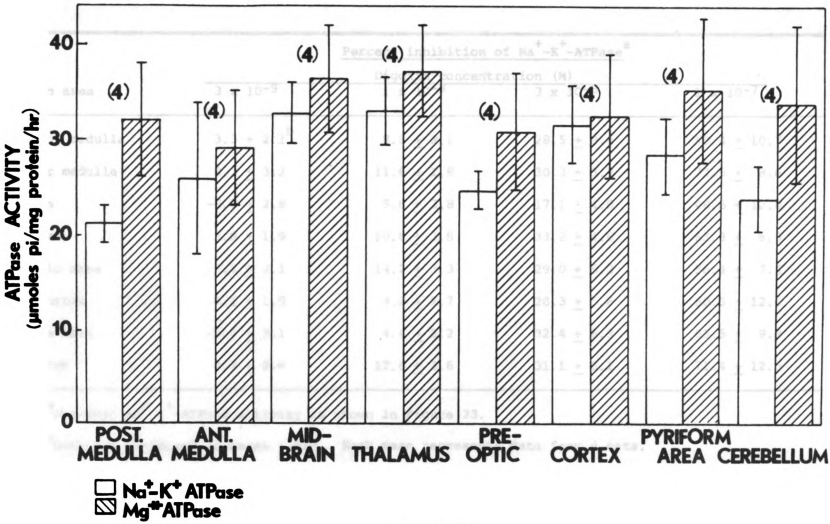
ATPase ACTIVITY
(μmoles pi/mg protein/hr)
40
30
20
10
0
(4)
(4)
(4)
(4)
(4)
(4)
(4)
POST. MEDULLA
ANT. MEDULLA
MID-BRAIN
THALAMUS
PRE-OPTIC
CORTEX
PYRIFORM AREA
CEREBELLUM
Na+-K+ ATPase
Mg++ATPase

Figure 23

Table 4. *In vitro* inhibition of cat brain Na^+-K^+-ATPase activity by digoxin

Brain area	Percent inhibition of Na^+-K^+-ATPase[a] Digoxin concentration (M)			
	3×10^{-9}	1×10^{-8}	3×10^{-8}	1×10^{-7}
Posterior medulla	3.3 ± 2.1[b]	8.0 ± 2.1	28.5 ± 9.4	50.4 ± 10.7
Anterior medulla	0.3 ± 3.2	11.6 ± 1.8	30.3 ± 3.7	72.5 ± 8.6
Midbrain	-0.9 ± 2.8	5.6 ± 3.8	17.1 ± 4.6	48.6 ± 12.0
Thalamus	5.8 ± 1.9	10.6 ± 3.8	33.2 ± 4.6	63.3 ± 6.5
Pre-optic area	7.2 ± 2.1	14.2 ± 3.3	29.0 ± 5.2	56.4 ± 7.3
Motor cortex	5.1 ± 1.9	9.2 ± 2.7	28.3 ± 5.6	60.3 ± 12.6
Pyriform area	-2.4 ± 3.1	4.4 ± 2.2	32.4 ± 6.3	63.5 ± 9.9
Cerebellum	4.3 ± 2.6	12.6 ± 3.6	31.1 ± 6.1	71.4 ± 12.5

[a]Pre-drug Na^+-K^+-ATPase activity is shown in Figure 23.

[b]Data are expressed as means ± SEM. Each mean represents data from 4 cats.

Figure 24. *In vitro* inhibition of Na^+-K^+-ATPase from 8 brain areas of the cat by digoxin.

The dose of digoxin and the % inhibition of Na^+-K^+-ATPase are plotted on logarithmic scales. Each line represents the inhibition of Na^+-K^+-ATPase in one brain area by digoxin. Four cats were used in this study; thus, the replication of each concentration for each brain area is 4. Each line was approximated by linear regression. The regression coefficients for these lines ranged between 0.58 and 0.85. Pre-drug Na^+-K^+-ATPase activity is shown in Figure 23.

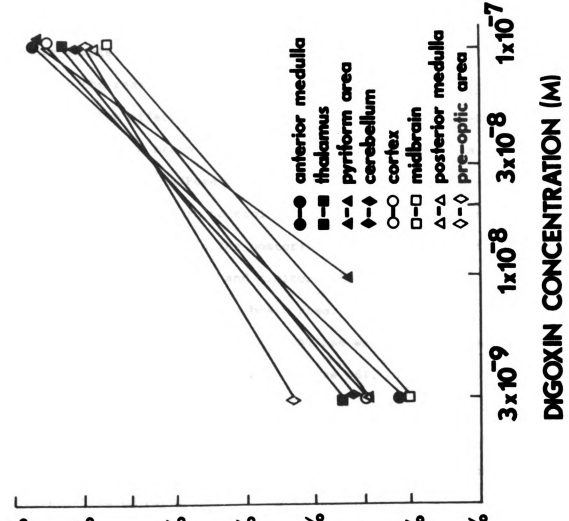
100%
50%
20%
10%
5%
2%
1%
% INHIBITION
anterior medulla
thalamus
pyriform area
cerebellum
cortex
midbrain
posterior medulla
pre-optic area
3x10⁻⁹
1x10⁻⁸
3x10⁻⁸
1x10⁻⁷
DIGOXIN CONCENTRATION (M)

Figure 24

Brain Na^+-K^+-ATPase from Cats Treated with Digitoxin

In the next series of experiments, saline or lethal doses of digitoxin were administered intravenously and Na^+-K^+-ATPase activity was determined in 8 brain areas. Digitoxin was used in these experiments since the complex of digitoxin with Na^+-K^+-ATPase is more stable than that of digoxin. The use of digitoxin thus minimizes the reactivation of inhibited enzyme during the preparative procedure. The effect of lethal doses of digitoxin on Na^+-K^+- and Mg^{++}-ATPase from 8 brain areas is shown in Figure 25. No significant inhibition of Na^+-K^+-ATPase was observed in any of the 8 areas. Na^+-K^+-ATPase activity in control (saline treated) and digitoxin treated cats was lowest in the posterior medulla. No significant differences between control and digitoxin treated cats were observed in Mg^{++}-ATPase activity in any brain area.

Ouabain binding to brain Na^+-K^+-ATPase was also compared in control and digitoxin treated cats (Figure 26). Treatment of the cats with digitoxin did not decrease ouabain binding in any area. Binding in the cortex was enhanced by digitoxin treatment. Ouabain binding was lowest in the posterior medulla in control and in digitoxin treated cats.

Figure 25. Effect of intravenous lethal doses of digitoxin on cat brain Na^{+}-K^{+}- and Mg^{++}-ATPase activity.

The data are expressed as means $\pm$ SEM. The numbers in parentheses represent the number of brains assayed. No significant inhibition of ATPase was detected in any brain area in the cats treated with digitoxin. Significant enhancement of Na^{+}-K^{+}-ATPase (indicated by asterisk) occurred in the cerebellum of treated cats.

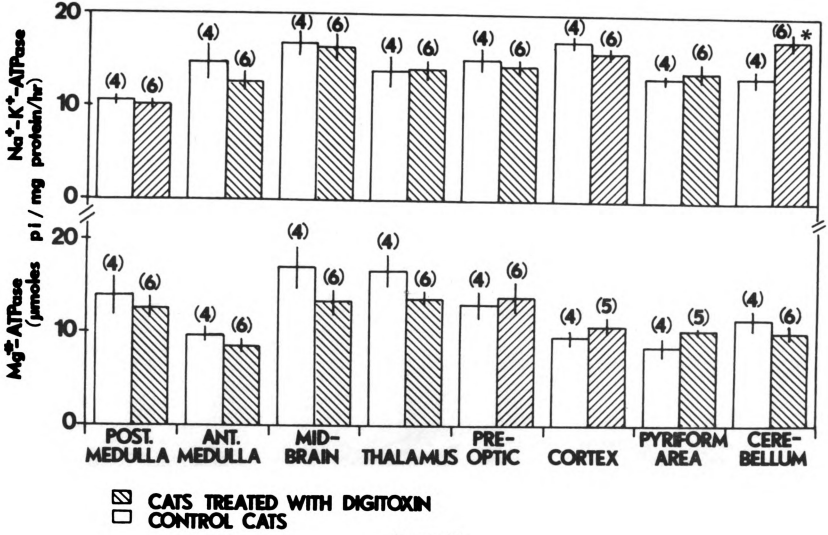
Na+-K+-ATPase
µl / mg protein/hr
Mg++-ATPase
(µmoles
20
10
0
(4)
(6)
(5)
*
POST. MEDULLA
ANT. MEDULLA
MID-BRAIN
THALAMUS
PRE-OPTIC
CORTEX
PYRIFORM AREA
CERE-BELLUM
CATS TREATED WITH DIGITOXIN
CONTROL CATS

Figure 25

Figure 26. Effect of intravenous lethal doses of digitoxin on (^{3}H)-ouabain binding to Na^{+}-K^{+}-ATPase from cat brain assayed *in vitro*.

Tritiated-ouabain binding was used to estimate the concentration of Na^{+}-K^{+}-ATPase unoccupied by digitoxin. The data are expressed as means $\pm$ SEM. The numbers in parentheses represent the number of brains assayed. No significant decrease in ouabain binding was detected in any brain area. Enhanced binding occurred in the cortex of treated cats (indicated by an asterisk).

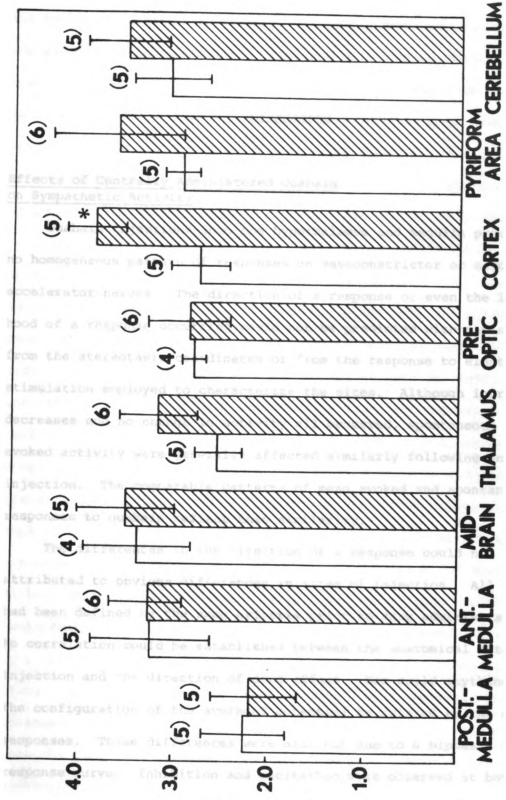
OUABAIN BINDING
picomoles/mg protein
4.0
3.0
2.0
1.0
(5)
(5)
(5)
(6)
(4)
(5)
(6)
(5)
(6)
(4)
(6)
(5)
*
(5)
(6)
(5)
(5)
POST.-MEDULLA
ANT.-MEDULLA
MID-BRAIN
THALAMUS
PRE-OPTIC
CORTEX
PYRIFORM AREA
CEREBELLUM
CONTROL CATS
CATS TREATED WITH DIGITOXIN

Figure 26

DISCUSSION

Effects of Centrally Administered Ouabain on Sympathetic Activity

Ouabain injections into the hypothalamus and medulla produced no homogeneous pattern of responses on vasoconstrictor or cardioaccelerator nerves. The direction of a response or even the likelihood of a response occurring could not be predicted with any accuracy from the stereotaxic coordinates or from the response to electrical stimulation employed to characterize the sites. Although increases, decreases and no change in activity all occurred, spontaneous and evoked activity were generally affected similarly following an injection. The comparable patterns of mean evoked and spontaneous responses to ouabain reflect this similarity.

The differences in the direction of a response could not be attributed to obvious differences in sites of injection. All sites had been defined by the same criteria prior to drug administration. No correlation could be established between the anatomical site of injection and the direction of drug effect. Nor could anything in the configuration of the averaged potential be related to the opposing responses. These differences were also not due to a biphasic dose response curve. Inhibition and excitation were observed at both low and high doses. As the dose was increased, a greater incidence of responses occurred and some responses became larger in magnitude,

but no reversal in the direction of responses occurred. In those experiments in which inhibition of nerve activity was particularly dominant, lower doses (1-10 ng) were tested but these also evoked inhibition. It is reasonable that central injections of ouabain had several effects on peripheral nerves, since Roberts (1970) and Roberts *et al.* (1974) described similarly complex patterns of effects on sympathetic nerves following intravenous administration of digitalis.

The divergent responses to ouabain were probably due to the anatomical complexity of the brain stem and hypothalamus. The reticular formation consists of a mosaic of afferent and efferent neurons connected in such a manner to allow multiple continuous and intensive interactions between various regions (Brodal, 1957; Scheibel and Scheibel, 1967). The hypothalamus is also an integrating center having multiple functional elements (Bard, 1960). This anatomical complexity has been reflected in functional studies of medullary pressor and depressor areas. Wang and Ranson (1939) constructed maps of depressor and pressor responses to electrical stimulation of the medulla and found considerable overlap of pressor and depressor sites. Chai and Wang (1962) extended these experiments further and found that moving the electrode only 1 mm could reverse a depressor to a pressor response. In the present study, in the process of identifying sympathetic excitatory sites as described in Methods, similar reversals of inhibitory to excitatory responses were encountered when the electrode was moved in 1 mm increments. Even in single hypothalamic sites, alteration of stimulus frequency has been reported to cause reversals of cardiovascular responses (Pitts *et al.*, 1941).

Considering this anatomical and functional heterogeneity of the medulla and hypothalamus, it is not surprising that injections of ouabain into these areas evoked a variety of responses. Similarly unpredictable responses to central injections were reported by Maillis (1974), who iontophoretically injected excitant amino acids into the cat cortex and observed responses from neurons which were beyond the anatomical range of direct drug effects. He suggested that these distant neurons were influenced transneuronally by interconnected neural elements.

As opposed to the discrete injections possible with microiontophoresis, microliter volumes of injected ouabain penetrated an area of 1.0-2.0 mm^3. Two microliters of black dye was injected at the end of some experiments and the area of stained tissue measured. The spread of 2 µl through 1.0-2.0 mm^3 correlates well with results of Hull *et al.* (1967), who conducted experiments to determine the area of brain tissue affected by various microinjection volumes. Thus, it is certainly feasible that drug concentration in an area this size could affect functionally different regions.

The lack of a clear dose-response relationship may also be related to the anatomical complexity of the brain. Drug effects on the peripheral nerve may have been the net result of effects of ouabain on central inhibitory and excitatory neurons. Differences in the balance of inhibitory and excitatory elements in the central sites of injection could have affected the direction or the magnitude of a drug response. The use of different sites for each injection of digitalis made subtle site to site differences a very real

complication in attempting to determine a dose-response relationship. There was suggestive evidence that increasing doses of ouabain produced an increasing effect on the peripheral nerve (Figure 3), but this relationship was not always apparent, either in an individual animal or in the combined data. Multiple doses injected into the same site might have provided a definitive dose-response relationship. This was attempted, but technical difficulties with the fine multibarrel electrodes and artifacts related to accumulating volumes and tachyphylaxis from multiple injections made interpretation of these experiments impossible.

It is interesting that a drug which directly affected only a small area of the medulla exerted profound effects on spontaneous vasoconstrictor and cardioaccelerator nerve activity with accompanying changes in blood pressure and heart rate. This presents evidence that alteration of the activity in a small area of medullary tissue may have considerable influence on resting blood pressure and heart rate.

The effects of hypothalamic ouabain on spontaneous activity are even more intriguing since the question of a tonic hypothalamic influence on resting sympathetic tone is a subject of great debate (Manning, 1965; Peiss, 1965; Smith, 1974). Redgate and Gellhorn (1956) injected barbiturates or procaine into the posterior hypothalamus and produced depression of blood pressure and heart rate. They suggested this to be evidence that tonic sympathetic impulses from the hypothalamus participate in the maintenance of resting blood pressure and heart rate. Hypothalamic injections of ouabain

which permeated a small area evoked significant changes in spontaneous activity in cardioaccelerator nerves. This, too, could be interpreted as a drug action in an area of the hypothalamus which generates peripheral sympathetic tone. However, it is also possible that ouabain simply evoked activity in hypothalamic sites which impinge on areas of tonic output.

The three sympathetic nerves tested each had a slightly different pattern of response to ouabain. Generally, decreases in activity were seen more frequently than increases. This tendency was exaggerated in the postganglionic inferior cardiac nerve when medullary injections evoked one increase in activity compared to 30 decreases. It is tempting to speculate that a ganglionic modulation such as occlusion might be responsible for the lack of increases in activity on this nerve. A considerable overlap of preganglionic fibers has been reported to occur in the stellate ganglion, thus allowing an extensive degree of occlusion (Koizumi and Brooks, 1972). Perhaps increases in preganglionic activity induced centrally by ouabain were masked by occlusion in the ganglion and thus not observed on the postganglionic nerve.

Following injections into the hypothalamus, inhibition of activity was most dominant in the preganglionic stellate nerves. Fewer increases in activity occurred on the preganglionic nerve than the postganglionic nerve. One could again attempt to relate preganglionic and postganglionic differences evoked from the hypothalamus to some ganglionic modification of preganglionic traffic. Since spatial summation also occurs in autonomic ganglia, perhaps

subliminal increases in preganglionic activity were magnified in the ganglion so that some increases became apparent on the postganglionic nerve. However, the differences in the responses of these two nerves to hypothalamic ouabain injections could as easily be coincidental. A small number of hypothalamic sites were tested while monitoring the activity of preganglionic nerves, and only a small proportion of these sites responded at all.

Although significant neural and cardiovascular changes occurred in response to central injections of ouabain, no incidence of arrhythmias was observed. Electrical stimulation of 24% of the medullary and hypothalamic sites of injection evoked arrhythmias, but even in these sites, ouabain did not induce abnormal cardiac rhythms. This is in contrast to the report of Bircher *et al.* (1963) and Basu Ray *et al.* (1972), who described cardiac arrhythmias following central injections of digitalis. Although exact causes of these discrepancies are unknown at this time, several possibilities exist. For example, Bircher *et al.* (1963) and Basu Ray *et al.* (1972) injected large doses of digitalis in contrast to the present study. Initial concentrations of digitalis in the brain resulting from intracranial injections of such large doses would appear to be much greater than those in cerebrospinal fluid (Garan *et al.*, 1973) or brain tissue (Dutta and Marks, 1966; Levitt *et al.*, 1973) following intravenous administration of the drug. Basu Ray *et al.* (1972) also injected relatively large volumes. Although 2 µl saline injections had little effect in the medulla, similar injections of saline into the hypothalamus caused rather large changes in evoked and spontaneous

cardiac nerve activity. Reducing the volume to 1 µl decreased the magnitude of the responses to saline injections but even this volume was still apparently large enough to cause mechanical or chemical disturbances of hypothalamic neurons. Alternatively, the small injection volume employed in the present study may have resulted in a failure of the ouabain injection to affect enough neurons simultaneously to evoke arrhythmias. This, however, is unlikely since electrical stimulation which appeared to have a similar magnitude of spread was capable of inducing arrhythmias in a substantial number of trials. Additionally, the type and depth of anesthesia may affect the response to centrally injected drugs.

Finally, one must question how central injections of ouabain produced these multiple effects on peripheral sympathetic activity. Did ouabain stimulate central neurons or did it depress them? Did it act similarly or in an opposite manner on excitatory and inhibitory cells? Considering the complex anatomy of the medulla and hypothalamus, it is certainly possible that ouabain had a common action on functionally different neurons to produce a diverse effect on peripheral nerve activity. Since cardiac glycosides have been shown to depolarize peripheral nerve fibers *in vitro* (Ritchie and Straub, 1957), and to increase their excitability *in vivo* (Ten Eick and Hoffman, 1969), it is plausible that within the dose range of 1-1000 ng, ouabain acted by exciting, not inhibiting, central neurons. Thus, the variety of responses evoked by central injections of ouabain could be attributed to stimulation of both excitatory and inhibitory influences on cardiovascular sympathetic outflow.

Similar digitalis induced enhancement of excitatory and inhibitory reflexes in the cat spinal cord has been reported by Osterberg and Raines (1973).

Effects of Intravenously Administered Digoxin on Sympathetic Activity

Effects of intravenously administered digoxin were compared in 4 groups of cats. The cats in these groups had different potential sites of drug action which could affect the observed nerve activity. This comparison revealed some primary sites of drug action in the sympathetic nervous system. It also indicated that some potential sites were apparently not of major importance. Inhibitory effects of digoxin on preganglionic or postganglionic sympathetic activity were observed only in cats with intact ninth and tenth cranial nerves. Since activation of the baroreceptor reflex inhibits sympathetic tone, this suggests that digoxin sensitizes or activates the baroreceptor reflex to cause depression of sympathetic discharge. This conclusion correlates well with reports from several laboratories (Heymans, 1932; Abiko *et al.*, 1965; McLain, 1970; Gillis, 1969). Since inhibitory effects were observed only in cats with intact afferent nerves and since McLain (1970) and Quest and Gillis (1971) have shown substantial increases in baroreceptor afferent discharge evoked by digitalis, the peripheral afferent component of the reflex is the likely site of drug action involved in neural inhibition. If other sites such as the central nervous system were involved in the inhibitory process, effects were either dependent upon intact baroreceptor afferents or were too subtle to observe in multiple

fiber nerves. The data are also not consistent with the induction of inhibition in the ganglion or in the peripheral sympathetic nerve fiber.

The large progressive increases in sympathetic activity to levels well above pre-drug activity were produced by digoxin only in postganglionic nerves. Significant increases were not observed in preganglionic nerves. However, preganglionic nerve activity tended to increase toward pre-drug activity levels as toxicity proceeded toward arrhythmia. It must be emphasized that at the same time blood pressure was falling in many of the cats and the increased sympathetic activity could have been produced reflexly from the baroreceptors. This complication makes it difficult to attribute these late increases in nerve activity toward pre-drug levels solely to direct neural effects of digoxin. Thus, the large progressive increases in sympathetic activity which were not complicated by baroreceptor reflex effects apparently stemmed from drug actions in the ganglion. The changes in preganglionic activity which occurred at toxic doses could have resulted from falling blood pressure or alternatively they could have been related to drug effects on chemoreceptors, the central nervous system or the peripheral nerve. However, since no changes in preganglionic activity occurred in response to digoxin after afferent input was eliminated, these data are not consistent with a primary site of drug action on the peripheral nerve or in the central nervous system.

If any significant drug action occurred in the central nervous system to excite sympathetic nerve activity, it must have been

dependent upon excitatory afferent input from the ninth and tenth cranial nerves. However, afferent baroreceptor influence on central sympathetic neurons is inhibitory. Although tonic excitatory chemoreceptor drive to respiration has been suggested (Heymans, 1951; Biscoe *et al.*, 1970), under conditions of normal pH, pCO_2 and pO_2, chemoreceptor influence on the circulation is negligible (Pelletier, 1972). Therefore, the increases toward control in preganglionic activity were probably due to falling blood pressure or to activation of peripheral chemoreceptors. Digitalis has been reported to activate peripheral chemoreceptors (Schmitt, 1958a,b). If chemoreceptor stimulation were involved in the late changes in nerve activity, a question arises regarding why digoxin excites baroreceptor fibers at low doses and affects chemoreceptor fibers only at high doses. Differences in size of the two groups of fibers could cause this pattern of responses to digoxin. The unmyelinated C fibers in the carotid sinus nerve (which digitalis could activate more readily) are predominantly baroreceptor in function (Fidone and Sato, 1969). The chemoreceptor fibers are primarily heavily myelinated A fibers which may only be activated by higher doses of the drug or longer exposure to it.

The lack of increases above initial preganglionic activity is in contrast to the reports of Gillis (1969), Gillis *et al.* (1972) and Pace and Gillis (1974). It is difficult to reconcile opposing data from different laboratories, but one explanation for the discrepancies relates to possible differences in experimental design. In the present study, after the onset of arrhythmia, injections of dextran were

given in an attempt to stabilize blood pressure. In spite of this treatment blood pressure fell. Without this intravenous volume expansion, blood pressure would have fallen even further and perhaps preganglionic activity would have increased above initial levels. Indeed, the sensitization of the baroreceptor reflex at toxic doses results in magnified sympathetic responses to decreases in blood pressure. McLain (1969) described dramatically exaggerated baroreceptor modulation of sympathetic activity following toxic doses of digitalis. This modulation, which was also observed in the present study (Figure 10), consisted of profound inhibition of activity in response to small increases in blood pressure and bursting enhancement of activity in response to small decreases in pressure.

The differing responses to digoxin observed in the present study on postganglionic nerves in cats with intact reflexes are similar to those reported by McLain (1969). They also compare favorably with the "non-uniform" responses on small filaments of the same postganglionic nerve described by Roberts *et al.* (1974). Results from both kinds of experiments indicate that digitalis enhances inhibitory or excitatory influences on postganglionic fibers. The differing responses of the whole nerves to digoxin in the present study could be a net result of opposing responses of individual filaments within the nerve to the drug. Perhaps the initial balance of inhibitory and excitatory influences on the postganglionic nerve determines the proportion of filaments inhibited or excited by digitalis. The direction of change of the postganglionic

nerve in the present study seemed to relate to the baroreceptor responsiveness of the cat before drug administration. Digoxin seemed to inhibit activity in cats which had strong reflexes and to increase activity in cats with less reactive baroreceptors.

In conclusion, digoxin had profound effects on nerves leading to the heart apparently by acting in the ganglion and on baroreceptor and perhaps chemoreceptor reflexes. Data from these experiments were not consistent with a primary site of drug action in the central nervous system or on the peripheral nerve fiber. However, digoxin may have acted in these areas to produce subtle changes in sympathetic activity which could not be detected with whole nerve recordings. Alternatively, another more lipid soluble or less polar cardiac glycoside which may have entered the brain more easily may have produced detectable central effects. Such a drug may also have penetrated the peripheral nerve myelin sheath to produce detectable effects. Chronic treatment with any of the cardiac glycosides likewise may have revealed central or peripheral nerve sites of drug action. Central effects of ouabain have been shown to excite or inhibit peripheral sympathetic nerves (Figures 3 through 9). This suggests that a central action of digitalis could contribute to the responses of sympathetic nerves observed after parenteral administration. However, the results from this study did not support the contention that the central nervous system or peripheral nerve axons are major sites of action of digoxin. Instead, the data suggest that digoxin exerts its profound neural effects primarily by acting on the ganglion and on the baroreceptor reflex.

Effects of Intravenously Administered Digoxin on Phrenic Nerve Activity

Phrenic nerve activity was enhanced by digoxin in cats with intact respiratory reflexes. This correlates well with the report of Gillis *et al.* (1972). However, an observation in the present study which contrasts with the results of other investigators (Sohn *et al.*, 1970; Yen and Chow, 1974) concerns arterial pCO_2 changes in response to digoxin. In many of the cats studied, digoxin appeared to increase end-tidal CO_2. Since the protocol in these experiments was to keep end-tidal CO_2 and arterial pCO_2 constant, any trends toward an increasing expired CO_2 or arterial pCO_2 were quickly reversed by small increases in respiratory rate. To keep end-tidal CO_2 and pCO_2 constant after administration of digoxin, the respiratory rate often had to be gradually increased. Sometimes a total increase of 2 RPM was needed during an experiment to keep expired CO_2 constant. This suggests that expired CO_2 and arterial pCO_2 would have increased in these cats. This tendency for end-tidal CO_2 to increase only occurred after digoxin administration. It did not occur in the absence of digoxin once nerve activity and the respiratory rate were stabilized. This change in end-tidal CO_2 contrasts with the reports of Sohn *et al.* (1970) and Yen and Chow (1974), who described decreasing arterial pCO_2 in response to ouabain. However, in their experiments, cats were either spontaneously breathing or artificially hyperventilated. The decreases in arterial pCO_2 described in their experiments occurred during the hyperventilatory effect of ouabain. Thus, it is quite likely that the decreased pCO_2 in response to digitalis was the result of hyperventilation. The cats in the

present study were paralyzed, artificially respired and incapable of hyperventilating to decrease pCO_2. This suggests that digitalis may cause an initial increase in pCO_2 which is then reversed by the hyperventilatory response to the drug, a response which was not possible in paralyzed cats. Perhaps an initial transient increase in pCO_2 is involved in initiating hyperventilation. This could not be the cause of continued respiratory enhancement since hyperventilation continues in the presence of low pCO_2, but perhaps an increase in pCO_2 may participate in the initial stimulus to respiration.

Since digitalis does not change central or arterial H^+ or K^+ concentration, pCO_2 or pO_2 in a manner which would continuously stimulate respiration, the drug apparently acts on neural structures through some other mechanism. In the present study, digoxin had no influence on phrenic nerve activity when afferent influences on respiration were eliminated. A similar lack of effect of digoxin on sympathetic preganglionic activity was observed in the absence of afferent input from the ninth and tenth cranial nerves (Figure 7). Therefore, the excitatory effects of digoxin on phrenic nerve activity were either due to peripheral drug actions or to central drug actions which were dependent upon afferent input. Excitatory effects could also have resulted from combined drug effects in both areas.

Digoxin could have acted centrally to subliminally excite respiratory neurons, producing an observable effect on phrenic activity only in the presence of peripheral excitatory drive from

afferent fibers in the ninth and tenth cranial nerves. Tonic peripheral chemoreceptor drive to respiration has been described (Heymans, 1951; Biscoe *et al.*, 1970) and excitatory inputs to respiration from lung stretch receptors travel in the afferent vagus (Larrabee and Knowlton, 1946; Reynolds, 1962). In the present study, a small amount of tonic drive to respiration was apparent in cats which were not treated with digoxin since phrenic activity in these cats decreased after sectioning their ninth and tenth cranial nerves. Changes in rate and amplitude of phrenic bursts could have resulted from subliminal excitation of central respiratory neurons in the presence of normal or enhanced afferent input. The presence of a subliminal drug effect could not be established or ruled out by the present experiments.

Alternatively, digoxin could have increased afferent drive to normally excitable central neurons to cause increases in phrenic burst amplitude and discharge rate. Digitalis does increase excitatory chemoreceptor afferent activity, making this an attractive hypothesis (Schmitt, 1958a,b). Afferent input was necessary for digoxin to evoke changes in both amplitude and rate of phrenic discharge. The changes in rate could have been due to Hering-Breuer reflex activation, resulting in increased rate secondary to increased inspiratory phrenic discharge. However, since cardiac glycosides increase respiratory rate (Sohn *et al.*, 1970) and phrenic discharge rate (Gillis *et al.*, 1972) in vagotomized cats, the increased rate can apparently occur in the absence of vagal reflexes.

Still other areas on which digitalis might act to increase respiration are the central respiratory chemoreceptors. These chemoreceptors are thought to exist on the ventrolateral medullary pial membranes (Loeschcke and Koepchen, 1958; Mitchell and Loeschcke, 1963). Respiratory responses may be evoked from these chemoreceptors similar to the emetic responses evoked by digitalis from the chemoreceptor trigger zone (Borison and Wang, 1951; Gaitonde *et al.*, 1965).

In summary, effects of digoxin on respiration depended upon intact afferent influences on respiratory neurons. Therefore, a primary site of drug action is not likely to be the brain stem respiratory neurons. Possibly, digoxin had a subliminal effect on central respiratory neurons which increased phrenic nerve activity only in the presence of excitatory afferent input. However, effects of digoxin on phrenic nerve activity were probably due to drug actions on peripheral sites on afferent nerves having excitatory influences on respiration.

Concentrations of Digitalis in the Central Nervous System Following Intravenous Administration and Their Effects on Na^+-K^+-ATPase

The concentration of digoxin in cerebrospinal fluid at the onset of arrhythmia (2×10^{-8} M) was approximately 10% of the total serum concentration and 59% of the free serum concentration. This CSF concentration compares favorably with the CSF concentration of digoxin in the dog reported by Garan *et al.* (1973). They administered 1.0 mg of digoxin intravenously and 15 min later detected 2.3 ng digoxin per ml CSF by radioimmunoassay techniques. The CSF digoxin concentrations in the present study are also in the same range as (^{3}H)-ouabain

concentrations in the cat brain reported by Levitt *et al.* (1973) or ouabain and digoxin concentrations in guinea pig and rat brain reported by Dutta and Marks (1966).

Randomly labeled (^{3}H)-digoxin was used in the first group of cats in the present study. Since tritium may dissociate from digoxin either in storage or after administration to an animal, the magnitude of this dissociation was determined by evaporating aliquots of radiolabeled drug and samples of CSF and serum filtrate containing digoxin. The residues were then reconstituted and analyzed by liquid scintillation spectrometry. Radioactivity was not lost by evaporating aliquots of (^{3}H)-digoxin. However, the CSF samples drawn at fibrillation lost 70% of the radioactivity after evaporation. Therefore, dissociation of tritium from digoxin probably resulted from metabolism of the drug in the cat. This problem makes it difficult to ascertain precise drug concentrations in the CSF in experiments in which randomly labeled drug was used. However, in later experiments, specifically labeled (12α-^{3}H)-digoxin was used. The concentrations of digoxin in CSF calculated from these experiments were quite similar to those concentrations calculated from the earlier experiments. It seems likely that as tritium dissociated from digoxin, a decrease in specific activity of the circulating digoxin occurred simultaneously. Apparently, when randomly labeled drug was used the error in the calculated CSF digoxin concentrations was minimal since the magnitude of tritium

released and the reduction in specific activity of the drug were similar.

The ability of these concentrations of digoxin to exert responses from central neurons is questionable. The concentrations of digitalis in the central nervous system in the present study and those reported by others (Dutta and Marks, 1966; Garan *et al.*, 1973; Levitt *et al.*, 1973) were smaller than the central concentrations which appear to be necessary to evoked cardiovascular or behavioral responses. Bircher (1963) evoked arrhythmias in the dog by injecting 32-48 µg of deslanoside into the fourth ventricle. Basu Ray *et al.* (1972) injected 20-80 µg of ouabain into the cat hypothalamus to evoke arrhythmias. Weinberg and Haley (1955) evoked cardiovascular responses in dogs by injecting 20-550 µg strophanthidin-K into the third ventricle. These doses would appear (at least transiently, if not for longer periods of time) to result in drug concentrations in certain areas of the brain or in the whole brain which were greater than those achieved after intravenous administration of drug. Since drug concentrations or distributions after central administration have seldom been verified, the concentration of drug available to central neurons in these experiments can only be estimated. The concentrations of digoxin in CSF also were much smaller than the minimal concentrations of ouabain used by the author in the central injection experiments to evoke responses on sympathetic nerves. Again the precise drug distribution after central injection was not known; thus, this comparison may not be valid.

Pharmacological responses to digitalis are often accompanied by inhibition of Na^+-K^+-ATPase in the affected organ. The inhibition is still measurable *in vitro* after the organ has been removed from the animal and the enzyme partially purified (see Introduction). However, no inhibition of Na^+-K^+-ATPase was observed in the brains of cats which had been slowly infused (intravenously) with lethal doses of digitoxin. Although the cats died in toxic arrhythmias, Na^+-K^+-ATPase was not inhibited in any area. Thus, it was apparently not inhibited in the brains of these cats during toxicity. One could argue that the enzyme had been inhibited *in vivo* and that the drug was dissociated from the enzyme during preparation. However, the evidence from earlier experiments (Donaldson *et al.*, 1971; Venturini and Palladini, 1973) and the high affinity of digitalis for brain enzyme (Tobin and Brody, 1972) makes this an unlikely explanation. The concentration of drug in the brain was probably not sufficient to inhibit Na^+-K^+-ATPase.

One complicating factor which must be considered is that 2×10^{-8} M digoxin did inhibit Na^+-K^+-ATPase activity approximately 10-20% *in vitro*. This conflicts with the results from animals treated *in vivo*. Such a conflict reflects the difficulty in extending conclusions regarding biochemical events occurring solely *in vitro* to the phenomenon occurring in a living animal. The *in vitro* inhibition of Na^+-K^+-ATPase may not accurately represent the interactions between digitalis and the enzyme which occurs in an intact animal. The conditions for *in vitro* assay of the effect of digoxin on Na^+-K^+-ATPase promoted a maximal enzyme inhibition. Sodium concentrations

in the incubation mixture were higher than intracellular concentrations of Na^+, and the ATP-dependent binding of digoxin to the enzyme occurred in the absence of K^+ during the preincubation period. Thus, the enzyme inhibition observed under this experimental condition represents the maximal inhibition obtainable with a given concentration of digitalis. In an animal, inhibition of Na^+-K^+-ATPase takes place in a less favorable environment containing the lower intracellular Na^+ concentration. Additionally, binding of digitalis to the enzyme occurs in the presence of extracellular K^+. Thus, the *in vivo* inhibition of Na^+-K^+-ATPase may be significantly lower than that observed *in vitro*. However, the possibility of a 10-20% inhibition of brain Na^+-K^+-ATPase in one or several areas during digitalis intoxication may not be excluded.

The physiological effect of such an inhibition in the brain is not known. In cats, brain Na^+-K^+-ATPase and cardiac Na^+-K^+-ATPase have similar sensitivities to cardiac glycosides. Brain enzyme in the present study was inhibited approximately 50% by 1×10^{-7} M digoxin (Figure 24). Repke (1965) showed that cardiac enzyme also is inhibited approximately 50% by 1×10^{-7} M ouabain or digitoxin. Since unbound serum digoxin concentrations were slightly higher than CSF concentrations in the present study, the effects on Na^+-K^+-ATPase and contractile force of the heart were probably greater than effects in the brain. A 20% inhibition of cardiac Na^+-K^+-ATPase is associated with a minimal effect on inotropy (Akera *et al.*, 1970). Therefore, even if intravenously administered digitalis did inhibit brain enzyme 10-20%, such an inhibition may not cause

significant pharmacological effects, whereas in the heart the higher concentration of digoxin would produce greater inhibition and hence a significant pharmacologic response. A similar relationship may not exist in the relatively digitalis insensitive species such as the rat (Repke, 1965), in which the brain Na^+-K^+-ATPase is markedly more sensitive to digitalis than cardiac enzyme. Large doses of digitalis produce profound central effects in the rat (Gold *et al.*, 1947). Such doses have little effect on the heart but produce significant inhibition of brain Na^+-K^+-ATPase in the rat (Gubitz *et al.*, 1973). This enzyme inhibition correlates well with the susceptibility of the rat to central effects of high doses of digitalis.

The regional differences in Na^+-K^+-ATPase in the present study compared favorably with other reports in the literature (Bonting, 1961; Fahn and Cote, 1968). The relatively low Na^+-K^+-ATPase activity in the posterior medulla correlated well with the low ouabain binding in the posterior medulla. The activity assay estimated the cation-dependent ATP hydrolyzing activity of the enzyme and the ouabain binding assay estimated the concentration of enzyme unoccupied by digitoxin. These two methods of evaluating the brain Na^+-K^+-ATPase usually compared quite favorably in all the brain areas.

In conclusion, the concentration of digoxin in cat cerebrospinal fluid at arrhythmia was approximately 2×10^{-8} M. This concentration of digoxin was capable of inhibiting brain Na^+-K^+-ATPase (10-20%) *in vitro*. However, Na^+-K^+-ATPase activity from cats which died in

digitoxin-induced ventricular fibrillation was not inhibited in any of the 8 brain areas examined. Perhaps the drug concentration in these animals was not sufficient to significantly inhibit brain Na^+-K^+-ATPase activity at the time of ventricular fibrillation.

SUMMARY AND CONCLUSIONS

Central injections of ouabain had diverse effects on the activity of peripheral sympathetic nerves. Injections into the medulla or hypothalamus evoked increases, decreases or no change in activity of the vasoconstrictor and cardioaccelerator nerves. The differing responses to ouabain could not be related to dose or to any identifiable differences in sites of injection. It appeared that ouabain was able to nonselectively exaggerate excitatory or inhibitory influences on sympathetic activity.

This study and previous investigations have shown that central actions of digitalis can influence sympathetic function when sufficient concentrations of the drug are in the brain. However, no evidence of central effects of digoxin were observed after intravenous drug administration. Digoxin evoked prominent responses in sympathetic nerves which could be attributed to drug actions in ganglia or baroreceptor afferent nerve fibers but no responses were seen which appeared to stem from direct drug actions in the central nervous system.

Excitatory effects of digoxin on phrenic nerve activity were dependent upon intact IX and X cranial nerves, again suggesting a peripheral afferent site of drug action. This correlated well with the effects of digoxin on sympathetic nerves.

The concentration of digoxin in the cerebrospinal fluid at arrhythmia was approximately 2×10^{-8} M. This concentration inhibited Na^+-K^+-ATPase *in vitro* only slightly. Treatment of cats with lethal doses of digitoxin had no inhibitory effect on brain Na^+-K^+-ATPase assayed after the cat died in toxic arrhythmias. These results also suggested that digoxin and digitoxin had little effect in the brain.

All the effects of moderate doses of digitalis described in these experiments and in the literature can be attributed to excitatory effects on nerve membranes. Even inhibition of sympathetic activity produced by digitalis was related to excitation of inhibitory processes. Digitalis also enhances spinal inhibitory reflexes. Digitalis appears to directly depress neural activity only at very high doses. This suggests a general action on all nerve membranes consistent with Na^+-K^+-ATPase inhibition. Moderate doses may inhibit Na^+ and K^+ pumping activity thus causing ionic imbalances to depolarize cells slightly and increase their excitability. But at much higher doses, ionic imbalances may become severe enough to decrease cellular excitability. The hypothesis that digitalis alters neural excitability by its action on Na^+-K^+-ATPase does complement the known biochemical and electrophysiological responses of nerve cells to the drug.

In conclusion, this series of experiments provided evidence that digitalis exerts its profound effects on sympathetic nerve activity by acting primarily on the ganglion and on baroreceptor afferent nerves. No biochemical or electrophysiological evidence

supporting central sites of drug action was obtained. Therefore, it was concluded that the primary sites of drug action on sympathetic nerves are in the peripheral nervous system and not in the central nervous system.

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