1982

Characteristics and Mechanisms of Norepinephrine Depletion from the Myocardium and Spleen of Endotoxic Rats

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CHARACTERISTICS AND MECHANISMS OF NOREPINEPHRINE DEPLETION FROM THE MYOCARDIUM AND SPLEEN OF ENDOTOXIC RATS

by

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

May

1982

ACKNOWLEDGMENTS

A debt of gratitude is extended to the members of the committee for their willingness to participate in this venture and for their suggestions and criticisms of this work. Many thanks to Dr. John X. Thomas, Jr. for his assistance and the use of his computer. Special gratitude is due my advisor and good friend, Dr. Stephen Jones, for sharing his guidance, patience, and half of his office with me throughout this endeavor.

VITA

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CHAPTER I

INTRODUCTION

Sympathetic nervous system activation is accepted as a general manifestation of circulatory shock. While early increases in sympathetic drive support the cardiovascular and metabolic adaptations to these stressful stimuli, excessive periods of increased sympathetic activity are considered detrimental, and contribute to the development of irreversibility. Indeed, blunting the actions of the sympathetic nervous system has beneficial effects in the outcome of circulatory shock. More specifically, protection with adrenergic blockade has been demonstrated in sepsis, both clinically and in various experimental models including endotoxin shock. Inappropriate sympathetic hyperactivation is, therefore, considered a major factor in the pathogenesis of endotoxin shock.

One critical issue regarding sympathetic nervous system activation in endotoxin shock is the methodology employed to quantitate nerve activity. Most demonstrations of increased sympathetic activity have been indirect in nature. These include increased circulating concentrations of norepinephrine and epinephrine as well as decreased tissue content of both bioamines. Interpretations of indirect neurochemical indices are limited. Neither marker of the sympathetic nervous system, i.e. circulating or tissue content of norepinephrine,

can adequately quantitate sympathetic activity. Furthermore, increased circulating norepinephrine may be due to reduced neuronal reuptake or decreased peripheral catabolism of the bioamine rather than increased sympathetic nerve activity. Depletion of peripheral tissue norepinephrine may be caused by inhibition of biosynthesis, decreased neuronal reuptake, or increased intraneuronal catabolism rather than augmented sympathetic nerve activation. Electronic recording of nerve activity provides a direct and quantitative assessment of sympathetic function. However, nerve recording requires invasive procedures on anesthetized animals and has provided conflicting results when applied to endotoxic models. Thus, although extensive work has implicated the sympathetic nervous system in the pathogenesis of endotoxin shock, few experiments have directly and quantitatively assessed sympathetic activity.

Two key, related aspects of sympathetic activation during endotoxin shock raise a second important issue. One is that norepinephrine depletion from tissues occurs during shock; a second is that blocking sympathetic activity is beneficial during endotoxic shock. Therefore, the depletion of norepinephrine transfers excessive amounts of the bioamine to areas accessible to adrenergic receptors, enhancing the effective sympathetic stimulation of the whole animal. Description of the characteristcs of the depletion and elucidation of the underlying mechanisms causing it are key issues to address if sympathetic dysfunction in endotoxin shock is to be understood and reversed. It may then be possible to apply the findings from experimental studies of

endotoxic shock to its clinical counterpart, and facilitate the management of sepsis.

Therefore, this dissertation: 1) describes the scientific evidence implicating the sympathetic nervous system in the pathogenesis of endotoxin shock, 2) reviews the normal mechanisms involved in maintenance of tissue catecholamine stores and the information known about the pathologic features of norepinephrine depletion in circulatory shock, 3) develops a logical approach to investigate sympathetic dysfunction in endotoxic shock, 4) presents findings pertinent to the questions raised, and 5) relates the present results to the existing literature.

CHAPTER II

REVIEW OF RELATED LITERATURE

A. Evidence for Sympathetic Activation in Endotoxin Shock

As outlined in the introduction, the sympathetic nervous system has been implicated in the pathogenesis of endotoxin shock. Although the demonstrations of sympathetic activity have been convincing, the methodologies employed have been qualitative and indirect in nature. Essentially four lines of investigation have provided evidence for sympathetic activation during endotoxin shock: 1) demonstration of increased plasma catecholamine concentrations, 2) depletion of tissue catecholamine stores, 3) radioisotopic experiments that traced the fate of injected radiolabeled catecholamines, and 4) direct electrophysiologic measurement of nerve activity. Each of these four areas will be reviewed in the following sections.

1. ELEVATION OF PLASMA CATECHOLAMINES

Plasma catecholamines have been measured in a variety of endotoxic models. Various results have been reported. In some models a singular rise in plasma epinephrine has been demonstrated. Spink et al. (142) measured femoral arterial catecholamine concentrations in five groups of endotoxic dogs. Increasing doses of endotoxin (Group I)

resulted in progressive increases in plasma epinephrine, but only a minimal increase of norepinephrine at the highest dose (2 mg/kg). The rate of endotoxin infusion (Group II) had the effect of raising epinephrine levels minimally. Rapid infusion of an endotoxin dose produced greater plasma epinephrine levels than slow infusion at only one time point (one hour) after infusion even though rapid infusion resulted in a 50% per cent higher lethality than slow infuson. Dogs tolerant to endotoxin (Group III; surviving an initial dose 3 weeks previously) demonstrated lower lethality than non-tolerant dogs, but both groups showed the same plasma epinephrine levels. Adrenalectomy (Group IV) or complete cervical spinal cord section (Group V) sensitized the dogs to endotoxin. The procedures also totally eliminated the elevated plasma epinephrine associated with endotoxin infusion. Thus, the experiments showed that endotoxin caused a selective increase in plasma epinephrine that was dose-dependent and neurally-mediated. The catecholamine response was subsequently shown by the same group of investigators to be more pronounced in puppies (124). Nykiel and Glaviano (112) in studies on anesthetized endotoxic dogs reported similar results. The experimental model was different to some extent. Blood was sampled from the adrenal vein to assess adrenal medullary function. The results indicated that epinephrine, but not norepinephrine was increased in adrenal venous blood of endotoxic animals. The increased epinephrine occurred only in the presence of decreased arterial blood pressure. Additionally, the epinephrine response was eliminated by section of the splanchnic nerves. Thus, the

increase in plasma catecholamines was again shown to be dependent on nerve activation.

Further investigation of adrenal medullary contribution to circulating catecholamines has been performed. Egdahl (35,36) studied the differential effects of endotoxin on adrenal medullary and cortical function in the dog. Adrenal venous blood was collected from indwelling catheters in unanesthetized dogs and analyzed for total catecholamines and 17-hydroxycorticosteroids to assess medullary and cortical function, respectively. Low doses of endotoxin (0.01 mg/dog) stimulated adrenal cortical secretion and fever but infrequently led to increased catecholamine levels. A higher dose of endotoxin (0.2 mg/10 to 18 kg dog; 10% lethality) resulted in adrenal medullary secretion as well as stimulation of the adrenal cortex and a febrile response. Spinal transection at C-7 abolished the catecholamine release but left the adrenal cortical and febrile response intact. Thus, differential effects on the adrenal medulla and cortex were observed. Endotoxin elicited a sympathoadrenal response that was presumably dependent on descending spinal input. The results of this study were generally confirmed by a subsequent study by Hokfelt (70). Prager et al. (122) investigated the role of adrenal corticosteroids in the adrenal medullary response to endotoxin. Adrenal vein output of catecholamines and systemic arterial catecholamine concentrations were measured in dogs treated with either methylprednisolone sodium succinate (30 mg/kg) followed by endotoxin (0.5 mg/kg; 60% lethality) or endotoxin alone. Endotoxin elicited increases in arterial and adrenal venous

norepinephrine and epinephrine. Dogs pretreated with the corticosteroid demonstrated lower norepinephrine and epinephrine output from the adrenal gland than dogs not given corticosteroids; arterial norepinephrine was the same and arterial epinephrine was lower in dogs treated with the corticosteroid. Thus, the study demonstrated that the sympathetic nerves are activated during shock and that the adrenal component of the activation (epinephrine output) could be suppressed by corticosteroid administration - an intervention shown to be beneficial for survival in shock.

Plasma catecholamine increases during endotoxic shock have also been shown to be blunted by other interventions. Feuerstein et al. (43) described increased plasma epinephrine and norepinephrine concentrations during two hours of endotoxemia in anesthetized cats. Prior injection with indomethacin, a prostaglandin synthesis inhibitor, attenuated the blood pressure decrease associated with endotoxin administration. The better hemodynamic status obtained with indomethacin also attenuated the norepinephrine and epinephrine increases. Devereux et al. (33) investigated the effect of heparin pretreatment on dogs in endotoxin shock. In control dogs injected with only endotoxin, plasma norepinephrine (superior vena caval cannula) but not epinephrine was increased. Heparin pretreatment effectively suppressed the norepinephrine increases. This report of no alterations in plasma epinephrine in endotoxic dogs with an intact splanchnic sympathetic supply is rare. Most studies have demonstrated that adrenal medullary secretion of epinephrine was extremely sensitive to the effects of

endotoxin. No speculation was made for the singular increase in plasma norepinephrine or the blunted norepinephrine response after heparin.

Other studies using similar dog models have demonstrated increased plasma concentrations of norepinephrine and epinephrine (66,126,127). Rosenberg et al. (126,127) found that both norepinephrine and epinephrine increased markedly after endotoxin injection (dose was only reported as a "lethal dose") in morphine-sedated dogs. Plasma samples were taken from systemic venous blood. The increased epinephrine levels soon returned to control level, but norepinephrine remained slightly elevated throughout the ten hour procedure. When animals approached the throes of death and blood pressure fell precipitously, both plasma norepinephrine and epinephrine rose dramatically. The inverse correlation between blood pressure and plasma catecholamines is similar to the report of Nykiel and Glaviano (112) cited earlier. However, not all studies that measured hemodynamic parameters as well as circulating catecholamine levels showed the same relationship. Heiffer et al. (66) have documented increased circulating norepinephrine and epinephrine (blood samples taken from the marginal ear vein) during the post-hypotensive phase of endotoxic shock in the rabbit. It was noted that during the initial hypotensive phase, the plasma catecholamines were not changed from control.

It is evident that many different results were obtained in the outlined studies. Some of the catecholamine responses were dependent on blood pressure responses; some were not. Some models responded to

endotoxin with increases in epinephrine or norepinephrine or both catecholamines. The different findings can be attributed to different animal species, different doses of endotoxin, different sensitivities to endotoxin within a given animal species, and also different locations for blood withdrawal. With respect to species differences, Hall and Hodge (62) performed a comparative study on the effects of endotoxin on plasma catecholamine and angiotensin levels in dogs and cats. The vasoactive hormones were measured by bioassay (contractile response) using a continuous superfusion of rat colon and stomach strips for measurement of angiotensin and catecholamines (total norepinephrine and epinephrine), respectively. In light of other methodologies available at the time of this study (1971) to measure catecholamines, one might question the validity of the methods. However, the control and calibration experiments seemed adequate and the study deserves mention because of its comparative nature. In dogs, an immediate rise in angiotensin was observed, followed by a later variable rise in catecholamines. In cats, an early rise in plasma catecholamines occurred followed by a later rise in angiotensin. The results of this study demonstrated the variability of circulating neurohormonal levels in different species in response to endotoxicosis. The endotoxin dosage was 2 mg/kg infused over a 5 to 15 minute period. No indication was made concerning differential sensitivity of the different species to endotoxin except the note that all dogs and 11 of 12 cats survived the 90 minutes of endotoxicosis observed.

Quantitatively, different results were observed in the above reports. However, the important aspect of the studies is that, viewed as a whole, the work constitutes a body of literature that indicates heightened sympathetic activity during endotoxic shock. Naturally this evidence is largely indirect in nature since increased plasma catecholamines may be brought about by changes in not only release, but also reuptake, and degradation of the bioamines. However, no matter what the mechanism may be for increased circulating catecholamines, they will activate postsynaptic receptors as well as, albeit less discriminately than, locally released catecholamines. The end result is a functional, if not actual, increase in sympathetic activity. However, the demonstration of increased plasma catecholamines does not answer the fundamental question: To what extent does activation of sympathetic nerves occur during endotoxin shock?

2. DEPLETION OF TISSUE CATECHOLAMINES

Terminals of neurons generally contain constant amounts of neurotransmitter greatly in excess of that necessary to respond to normal bursts of electrical activity. This is true in the case of sympathetic post-ganglionic neurons. Even under high stimulation rates the level of norepinephrine in sympathetic nerve endings (and hence in sympathetically-innervated tissues) normally remains constant. Depletion of the norepinephrine reserve can occur in a number of circumstances, as will be discussed in a later section, and generally indicates sympathetic dysfunction or intense hyperactivation of the

nerves. Unless synthesis of norepinephrine is blocked, norepinephrine depletion is usually interpreted as increased release or transfer of the neurotransmitter out of the neuron and into the interstitium. In endotoxin shock the occurrence of depressed tissue levels of catecholamines concomitant with increased circulating levels of the same has been interpreted as strong evidence for increased sympathetic drive.

The first mention of depressed tissue catecholamine levels in endotoxin shock was by Dennis in 1939 (32). In a two page article, Dennis described the general reaction of laboratory animals (rabbits, guinea pigs, mice, and rats) to trichloroacetic acid extracts of defatted typhoid bacilli. Gross observations were reported during both the shock period and during necropsy. Within the discussion of the microscopic findings a single statement was made concerning catecholamine depletion in the adrenal glands: "The adrenals showed rapid loss of chromaffin material 3 hours after injection." The first report that focused on decreased tissue content of norepinephrine in endotoxic shock was by Heiffer, Mundy, and Mehlman in 1959 (25) and 1960 (113). The brief summary described the effect of one or six hours of E. coli endotoxin administration (5 mg/kg) on the catecholamine content of the adrenal glands of rabbits. One hour after endotoxin the epinephrine content was significantly reduced; the norepinephrine content remained unchanged. After six hours, however, adrenal epinephrine content was reduced 68% and norepinephrine content was reduced 44% from time-matched, saline-injected, control rabbits.

Although the first reports were made years earlier, Fine and his associates were the first group of investigators to study this phenomenon in detail (113,114,120,164,165). One study used dogs subjected to either intravenous or cerebral intraventricular endotoxin (164). Both routes of endotoxin administration resulted in 100% lethality. Catecholamines were analyzed by the trihydroxyindole fluorometric method. Four to seven hours after intravenous endotoxin injection, hearts, livers, and spleens were depleted of norepinephrine by 49%, 44%, and 22%, respectively. Intraventricular endotoxin (death occurred within ten to fourteen hours in all animals) caused decreased cardiac, hepatic, and splenic norepinephrine content of 31%, 17%, and 22%, respectively. Additional work by the same researchers demonstrated that denervation of the abdominal viscera protected against endotoxic shock (114,165) as well as hemorrhagic shock (114,164,165). The celiac and superior mesenteric ganglia were surgically denervated in both dogs and rabbits (114). Animals were allowed three to eight weeks to recover before undergoing endotoxin administration. Both species were treated with endotoxin intravenously; endotoxin injection into cerebral ventricles was performed in another group of dogs, and additional rabbits were injected subdurally with endotoxin. Denervation of the abdominal viscera significantly increased survival rate (surviving animals were sacrificed four to twelve days after the shock procedure) in all models except those rabbits injected intravenously with endotoxin. The protection in dogs was far more dramatic than in rabbits. Hemodynamic data were presented that demonstrated better perfusion to the splanchnic area in denervated animals. The authors

attributed the protection to the sparing of liver and reticuloendothelial function. Their conclusion was that chronic surgical denervation of the splanchnic nerves decreased both the release of norepinephrine and the subsequent vasoconstriction and flow deprivation to the liver and intestine which resulted in better function of these organs.

Fine and associates also demonstrated that acute denervation of the abdominal viscera protected against the norepinephrine depletion during endotoxic shock (165). One half of the nervous supply to spleens of dogs was interrupted acutely prior to endotoxin administration. During shock, the denervated hemi-spleens were not depleted of norepinephrine, continued to contract in response to exogenous norepinephrine, and showed no histopathologic changes compared to the innervated half-spleens. Throughout these studies, the fundamental conclusion drawn by the investigators was that the depletion of norepinephrine was caused by massive release of the catecholamine (in a rate exceeding the ability of the neuron to synthesize it). The importance of the depletion was that the action of the liberated norepinephrine damaged the splanchnic organs and the myocardium and was thus one of the major mechanisms responsible for the pathogenesis of shock.

Pohorecky, Wurtman, Taam, and Fine demonstrated norepinephrine depletion in the endotoxic rat for the first time in 1972 (120). Five hours after various doses of endotoxin (1 to 5 mg endotoxin per 200-250

gm rat, intraperitoneally) norepinephrine content was measured in heart, spleen, and adrenal glands. A positive dose-response relationship was found - i.e. the greater the endotoxin challenge, the greater the norepinephrine depletion. Additional experiments showed that for a given dose of endotoxin (4 mg per rat) the norepinephrine depletion in spleen and adrenals was proportional to the length of time of endotoxicosis.

Recently, Bolton and Atuk, in a study of the effects of the sympathetic nervous system on endotoxin-induced lethality in rabbits, demonstrated norepinephrine depletion in hearts, kidneys, and lungs (9). Endotoxin was administered to rabbits (1.5-2.0 kg) at two times: an original slow intravenous injection of 0.4 mg followed 18 hr later by a 0.2 mg intravenous injection. Approximately 30% of the animals died within 18 hours of the initial injection; 30% died within 24 hours of the second endotoxin injection. The remaining rabbits were killed 24 hours after the second injection. When animals dying at all times were analyzed together, norepinephrine depletion was evident in heart, kidney, and lung. The long term effect of endotoxin on norepinephrine depletion in surviving animals could potentially have been studied if the authors had grouped the animals according to time of death.

Thus, norepinephrine depletion has been demonstrated in a variety of endotoxic animal models. The work of Fine and associates has shown that the depletion was avoided if the nervous supply to the organ in question was sectioned or blocked with an anesthetic. These results

suggested that the norepinephrine depletion was dependent on nerve-stimulated release and that endotoxin did not act directly on the nerve terminals to elicit transmitter release. Thus, the mechanism of depletion must be subsequent to nerve stimulation. However, the magnitude of sympathetic activation in shock is still in question. Some of the above and other studies investigated the mechanism of norepinephrine depletion and will be reviewed in a latter section.

3. RADIOISOTOPIC STUDIES

Radioisotopic labeling of neurotransmitters and their incorporation into existing transmitter pools enable design of experiments that reveal much more information than measurement of endogenous transmitter levels in tissue or plasma. Several studies have been performed in endotoxic models and have suggested that sympathetic activity was increased. However, while a potentially powerful tool was utilized, the experimental design limited the interpretation of the results.

The study of Pohorecky et al. (120) that described a time- and dose- response relationship between endotoxin and norepinephrine depletion also investigated catecholamine metabolism using radiolabeled norepinephrine. One hour before endotoxin injection (intraperitoneally) rats were injected intravenously with ^{3}H-norepinephrine to allow the labeled transmitter to be taken up by sympathetic nerve endings and equilibrate with the endogenous pool. Different doses of endotoxin were

injected and five hours later hearts, spleens, and adrenal glands were analyzed for ^{3}H-norepinephrine content. The experiments demonstrated that endotoxin (in a dose-dependent manner) reduced the amount of ^{3}H-norepinephrine that remained in the tissues in question. Also, similar experiments were performed to test the effect of one dose of endotoxin (4 mg/rat) at hourly intervals (up to five hours after endotoxin) on the ^{3}H-norepinephrine content of tissues. A time-dependent decrease in labeled norepinephrine was observed. The results were interpreted as indicative of increased norepinephrine turnover and hence increased sympathetic activity during endotoxicosis. Kinetic analysis of ^{3}H-norepinephrine decay in tissues to measure norepinephrine turnovers requires a different experimental design than used in the study. In the dose-dependent experiments endogenous norepinephrine as well as labeled norepinephrine decreased. It is difficult to interpret the results of various endotoxin dosages at one time after endotoxin injection. One might interpret the results as a dose-dependent effect of endotoxin to inhibit synthesis and increase degradation with no effect on norepinephrine release. Inhibition of norepinephrine synthesis would result in parallel decreases in endogenous and labeled norepinephrine; increased degradation due to endotoxin could also, in a dose-dependent manner, cause a parallel decrease in both labeled and unlabeled norepinephrine in a tissue. In the other set of experiments the time-dependent decrease in both endogenous and labeled norepinephrine (using one dose of endotoxin) demonstrates very little. No time-matched control series were performed to determine a normal decay of labeled norepinephrine in the tissues.

Additionally, because the endogenous content of norepinephrine also decreased over the five hours of the experiment, one might make the interpretation that endotoxin simply inhibited norepinephrine synthesis. Thus, both endogenous and labeled norepinephrine fell in parallel and no changes in specific activity were noted.

A study by Rao et al. (123) investigated norepinephrine metabolism in the endotoxic dog using ^{3}H-norepinephrine. Endotoxin (2 mg/kg) was administered to dogs 30 minutes after injection of ^{3}H-norepinephrine. Femoral artery blood samples were drawn 3, 5, and 30 minutes after endotoxin administration. Plasma ^{3}H-norepinephrine concentrations were 63%, 90%, and 75% of pre-injected controls, respectively. Analysis of ^{3}H-normetanephrine at the same times disclosed labeled metabolite increases of 48%, 92%, and 102% at the indicated times. Labeled norepinephrine and normetanephrine were also measured in individual organs. ^{3}H-norepinephrine in atria, left ventricles, and kidneys was unchanged 30 minutes after endotoxin injection; the levels in spleens and adrenal glands were increased. In all organs, labeled normetanephrine increased. Vague conclusions and interpretations were made: The authors suggested that increased release and metabolism of catecholamines had occurred. Little conclusive information was gained by this study.

Cavanagh et al. (16) measured numerous hemodynamic, hematologic, and neurohumoral parameters in a primate model of endotoxicosis. Plasma norepinephrine was assessed by quantitating the amount of

^{3}H-norepinephrine present in blood after an endotoxic challenge (7 mg/kg). Prior to endotoxin, the labeled amine was injected into the baboons, allowed to be taken up in sympathetic nerve endings, and equilibrate with the endogenous pool. The radioactivity in the plasma associated with norepinephrine increased 50% within 3 minutes of endotoxin injection. The ^{3}H-norepinephrine level in plasma then decreased with time and was no different than control by two hours after endotoxin. This study did not quantitate the actual norepinephrine concentration in plasma, and also failed to quantitate the amount of ^{3}H that was associated with the products of norepinephrine metabolism. The level of ^{3}H-norepinephrine in the plasma is dependent on release and degradation of the bioamine; it is also dependent on the catabolism of labeled amine within the nerve terminal prior to its release. Interpretation of the data is difficult. The conclusions of increased sympathetic activity may be entirely correct, but the experimental design does not allow more than a vague, qualitative conclusion to be made.

Conclusions from the above studies have indicated increased sympathetic drive during shock. Indeed, the study of Pohorecky (120) has been widely quoted in the shock literature as demonstrative of increased norepinephrine turnovers during endotoxic shock in the rat. However, closer inspection of the design of the studies raises questions regarding the strength if not the validity of the conclusions.

4. ELECTROPHYSIOLOGICAL STUDIES

The most direct method to assess sympathetic nerve function is to measure it directly by electrophysiologic methods. Several studies have employed this approach to assess sympathetic function in the endotoxic cat and dog, but the results of the different studies are conflicting.

Halinen (61), studied early effects of endotoxin (up to 15 minutes after administration) on cardiac efferent nerve activity in chloralose-anesthetized acid-base-controlled dogs. He reported increased cardiac postganglionic sympathetic activity for the duration of the experiment compared to control. At the time, aortic pressure had dropped markedly. Concomitantly, aortic arch baroreceptor activity was decreased. Unexpectedly, cervical vagal efferent activity increased after endotoxin administration. It was concluded that sympathetic pathways were functional early after endotoxin administration and that the increased discharges were primarily reflex in nature to maintain blood pressure. Endotoxin was speculated to modify baroreceptors, mechanoreceptors, or chemoreceptors. It was proposed that vagal tone increased after endotoxin because of chemoreceptor activation. A group of dogs was additionally given a small dose of pentobarbital to examine the possible modifying effects of the anesthetic on the results. The essential results were that the pentobarbital accentuated both the increased cardiac sympathetic efferent and cervical vagal efferent

activity. The modification was performed because many previous hemodynamic studies had used pentobarbital as an anesthetic.

In contrast to the work of Halinen (61), two reports in abstract form by Koyama et al. (86) and Santiesteben et al. (130) in the same laboratories, indicated that preganglionic splanchnic nerve activity was decreased after endotoxin injection in the chloralose-anesthetized cat even though blood pressure was significantly reduced. At no time during the 60 minute experiment was nerve activity increased over normal. Preganglionic splanchnic nerve activity was increased, however, in response to hypotension induced by hemorrhage in the same animal model (86). The authors concluded that arterial baroreceptors did not buffer endotoxic hypotension as effectively as hypovolemic hypotension.

Autonomic afferent function has also been assessed before and after administration of endotoxin (149). Baroreceptor discharge frequency was measured as a function of intrasinus pressure using an isolated sinus model in the dog. The relationship of discharge frequency versus pressure was shifted to the left indicating that the baroreceptors were firing at a higher rate for a given pressure stimulus. The conclusion was that the baroreceptors were reset to maintain blood pressure at a hypotensive level. It would then be consistent with these results that baroreceptor-stimulated sympathetic efferent activity was depressed in endotoxicosis.

Nerve recording would be expected to yield the most direct and clearest results. However, investigations during endotoxin shock have not been conclusive. Differences in the studies may be attributed to differences in species or anesthesia. Additionally, the experiments of Koyama and Santiesteban measured sympathetic preganglionic activity from the splanchnic region. Halinen measured sympathetic postganglionic cardiac nerve activity. It should also be noted that in all cases an anesthetized preparation was used which can easily modify autonomic nerve activity. In summary, no clear conclusions can be drawn from the existing literature on direct sympathetic nerve recording during endotoxin shock.

B. Evidence Supporting the "Pathogenetic" Properties of the Sympathetic Nervous System in Endotoxin Shock

The role of sympathetic nervous system activation in the pathogenesis of endotoxic shock has been extensively investigated and tested. Probably the first proposal for an autonomic mechanism in the action of endotoxin was by Reilly and associates, a group of French investigators, in 1935 (125). According to Thomas in a 1954 review on the physiological disturbances produced by endotoxins (147), the "Reilly phenomenon" received relatively little attention in American scientific literature. The theory held that there was a "selective affinity of endotoxin for sympathetic nervous system tissues." Evidence that supported this concept included the effect of small amounts of endotoxin injected in the vicinity of the splanchnic nerve that caused hemorrhage

and necrosis in the splanchnic region, shock, and death. Even larger amounts of endotoxin injected intramyocardially or by other routes had no effect.

The production of synthetic analogues of norepinephrine and epinephrine in the 1950's spurred much research in this area. Through the 1950's, therapeutic augmentation of sympathetic activity during all types of circulatory shock was an accepted clinical procedure (97). However, not all evidence supported this concept. Although adrenergic agonists were capable of maintaining blood pressure during the early phases of shock (154), overall survival was not improved (96,136). Zweifach et al. (167) demonstrated that endotoxin sensitized blood vessels to the effects of catecholamines. This finding was not confirmed in a similar preparation, however (104). Injection of epinephrine intradermally was shown to cause hemorrhagic necrosis when either preceded or followed by endotoxin injection (148). Indeed, infusion of catecholamines alone were shown to cause a shock-like state (49,103,158,161). Thus, by the 1960's, the foundations for a new working hypothesis regarding the protective effects of sympathetic blockade during shock were formed. Interestingly, one of the first reports of adrenergic blockade during endotoxicosis was as early as 1950 by Boquet and Izard (10). Dibenzyline, an alpha adrenergic antagonist, reversed the vascular constriction of rabbit ears to typhoid endotoxin. However, the administration of dibenzyline after endotoxin did not affect the lethality of the syndrome. The authors concluded that endotoxin liberated epinephrine or epinephrine-like substances which

caused intense vasoconstriction that could be blocked by sympathoadrenal antagonists. Major contributions by Nickerson and Lillehei documented the beneficial effects of blunting the actions of the sympathetic nervous system. It is at this juncture that pertinent literature of this area will be reviewed.

Gourzis, Hollenberg, and Nickerson (58) confirmed in rabbits the findings of Zweifach et al. (167) that endotoxin increased the pressor response to epinephrine and norepinephrine. Additional studies demonstrated that isotonic contractions of aortic strips incubated in vitro with endotoxin and whole blood were increased in response to endotoxin. No such increase was noted if blood or its constituents were replaced with a buffer solution. The Nickerson study also tested the lethality of endotoxin in rabbits and mice with and without two agents that blunt adrenergic responses. Results indicated that pretreatment with reserpine (causing depletion of endogenous norepinephrine stores) or phenoxybenzamine (blocking alpha adrenergic receptors) significantly decreased endotoxic lethality in both species. Pretreatment of mice with phenoxybenzamine doubled the dose of endotoxin necessary to induce a 50% lethality.

In a landmark publication Lillehei and MacLean (96) demonstrated the benefit of adrenergic blocking agents in canine endotoxin shock. Adrenergic agonists were shown to sensitize dogs to lethality in their endotoxic model (84% lethality with endotoxin alone; 100% lethality with norepinephrine or metaraminol given concomitantly with endotoxin).

Metaraminol also potentiated the effect of a sublethal dose of endotoxin (measured as an increase in lethality). Pretreatment with either chlorpromazine or phenoxybenzamine was used to block the vasoconstrictor effects of catecholamines released during endotoxicosis. All of the dogs of both these groups survived (Seventy-two hours after endotoxin dogs were considered permanent survivors.). On autopsy, animals treated with adrenergic antagonists showed normal bowel morphology and only moderate liver congestion and renal tubular damage compared to the endotoxic controls. Subsequently, a hemodynamic mechanism to explain the beneficial effects of adrenergic blockade in a similar model was investigated (73). Results demonstrated that alpha adrenergic blockade augmented venous return in dogs treated with endotoxin by decreasing the pooling of blood in the liver and other areas. The initial blood pressure drop after endotoxin was eliminated by phenoxybenzamine, but pressures were comparable in both groups by thirty minutes after endotoxin administration. Alpha blockade had no effect on heart rate, blood pH, or hematocrit.

More recently, the combination of a prostaglandin synthesis inhibitor, indomethacin, and phenoxybenzamine has been shown to increase survival of endotoxic rats (57). This effect was attributed to the superior hemodynamic status of the treated rats. The increased survival of endotoxic rats pretreated with alpha adrenergic blocking agents has also been shown to be associated with improved metabolic homeostasis. Filkins (46) demonstrated that alpha adrenergic blockade prevented the depression of hepatic gluconeogenesis associated with endotoxic shock

and protected against endotoxic hypoglycemia. However, the same study reported that beta adrenergic blockade sensitized rats to endotoxic lethality, depression of hepatic gluconeogenesis, and endotoxic hypoglycemia. A subsequent study by Kaelin and Rink (82) measured hepatic oxygen supply in the same model. They ascribed the improved metabolic status of the alpha blocked endotoxic rat to the ability of phentolamine to increase hepatic oxygen supply through its vasodilating properties.

Attempts have been made to blunt adrenergic vasoconstriction in endotoxic shock by making animals tolerant to the effects of catecholamines (34,71). Tolerance can be accomplished by injecting animals regularly with sublethal doses of epinephrine; subsequent injection with normally lethal doses of epinephrine does not result in death (37,152). In their study, Dietzman et al. (99) demonstrated that while 10% of control dogs survived an endotoxin challenge, 100% of epinephrine tolerant dogs survived the same dose of endotoxin. Data were presented to demonstrate the improved hemodynamic status of epinephrine tolerant dogs. Hruza et al. (71) produced blunted adrenergic responsiveness in rats by injecting epinephrine or norepinephrine suspended in oil 30 minutes prior to endotoxin injection. The procedure significantly protected rats against endotoxic lethality. If rats were previously eviscerated, the pretreatment did not affect lethality, implicating visceral vasoconstriction in endotoxic shock. It was concluded that the slowly diffusing catecholamines induced a decreased responsiveness to adrenergic stimuli or "autoregulatory

escape" before endotoxin was administered and thereby afforded protection. The importance of splanchnic vasoconstriction has been stressed by Fine previously (47), and the beneficial effects of splanchnic denervation have already been discussed in a previous section (114,164,165).

Finally, sympathectomy has been shown to have variable effects prior to endotoxin shock (2,9,58,99,150). Chemical sympathectomy in newborn rats (within the first few hours of birth) by either guanethidine, bretylium tosylate, or 6-hydroxydopamine did not affect survival after endotoxin injection compared to non-sympathectomized control rats (2). The lack of protection in newborn rats may have occurred because the sympathetic nerves were not fully developed at that time. Therefore, even the non-sympathectomized control rats may have had negligible adrenergic responses to endotoxin. 6-Hydroxydopamine pretreatment did afford protection (9) against endotoxin lethality in adult rabbits (64% control rabbits died; 25% of the sympathectomized rabbits died). Sympathectomy with reserpine in rabbits and mice also decreased lethality associated with endotoxin administration (58). Spinal block by epidural anesthesia sensitized dogs to hemodynamic demise and lethality in endotoxin shock (99). Because blood pressure fell precipitously after endotoxin in the spinal group, the authors concluded that total lack of sympathetic vasoconstriction was detrimental in endotoxicosis. However, sympathectomy by complete spinal blockade lacks a great deal of specificity and compromises hemodynamic status much more than sympathetic chain extirpation. Chemical

sympathectomy in dogs with 6-hydroxydopamine in the presence of adrenal demedullation also did not affect cardiopulmonary responses compared to dogs treated with endotoxin alone (150). However, 6-hydroxydopamine sympathectomy was not investigated in the presence of intact adrenals.

In addition to the literature cited showing no beneficial effects of sympathectomy during endotoxic shock, many reports describe the lack of protective affects afforded by adrenergic blockade in shock (63,69,87,102). Hinshaw and associates are of the strong influence that catecholamines and sympathetic activation are not a primary cause of pathologic hemodynamic changes during endotoxicosis, but that they may be secondary contributors to the splanchnic pooling and pre- and post-capillary resistance changes (69,87,102). In this regard, splanchnic denervation and adrenalectomy (69), alpha adrenergic blockade (102), and extirpation of the sympathetic chain and vagi (87) in dogs subsequently treated with endotoxin afforded no protection. Combined alpha and beta adrenergic blockade in the dog also did not afford any protective effects on hemodynamics during endotoxemia (63).

However, in the majority of cases, blunting of the sympathetic nervous system has been shown to protect against the pathogenesis of endotoxic shock. Evidence indicates that alpha adrenergic activation in shock compromises both hemodynamic and metabolic homeostasis. Conversely, beta adrenergic stimulation has not specifically been shown to be detrimental and through its vasodilating properties may actually be hypothesized to protect against the pathogenesis of endotoxin shock.

C. Tissue Norepinephrine Levels - Physiological and Pathological Processes

Depletion of norepinephrine from tissues has been reported in a variety of circumstances. Pharmacologic manipulation of catecholamine metabolic processes that inhibit synthesis (140) or vesicularization (106) of norepinephrine depletes the transmitter from nerve terminals. Also, chemical sympathectomy with 6-hydroxydopamine (107,121) or guanethidine (44) displaces norepinephrine from nerve terminals thereby depleting them of transmitter. Pathophysiological situations have also been shown to deplete norepinephrine. Clinically, norepinephrine is reduced in cardiac muscle from patients in congestive heart failure (18,21). In experimental models of cardiovascular disease, norepinephrine depletion has been documented in: 1) hearts of dogs (20) and rodents (131) in surgically induced chronic heart failure, 2) hearts and spleens of hamsters with genetic cardiomyopathies (138,139), and 3) canine hearts after left anterior descending artery occlusion (166). Acute induction into hypothermia (81) or chronic cold exposure in rodents (80) lowers norepinephrine levels in peripheral tissues. Immobilization stress also activates the sympathetic nervous system and causes cardiac norepinephrine depletion (139). Evidence has already been presented that documents norepinephrine depletion from both central nervous and peripheral tissues during endotoxin shock. In hemorrhagic shock the same phenomenon has also been reported (23,30,40,52,55,68).

Basic knowledge of the physiologic processes that regulate norepinephrine levels in tisses is a prerequisite for: 1) effective evaluation of literature addressing the pathologic changes underlying norepinephrine depletion during endotoxicosis and 2) design of experiments that further investigate the phenomenon. A brief review of the factors that determine norepinephrine content in peripheral tissues under normal conditions will first be presented. A survey of circulatory shock literature reveals that norepinephrine depletion in tissues is not confined to endotoxic shock. As mentioned, hemorrhagic models of shock have been shown to induce similar situations of norepinephrine depletion. Indeed, some reports have simultaneously investigated the depletion in both endotoxic and hemorrhagic models. Because knowledge of norepinephrine depletion during hemorrhage can shed light on possible mechanisms of endotoxin-induced norepinephrine depletion, and because many of the vascular lesions are similar in both of these types of shock, the literature addressing norepinephrine depletion during hemorrhagic shock will also be reviewed. No literature review of norepinephrine depletion in other types of circulatory shock will appear because the phenomenon has not been extensively investigated. Finally, the existing literature that addresses possible mechanisms of catecholamine depletion in endotoxin shock will be reviewed.

1. MAINTENANCE OF TISSUE NOREPINEPHRINE LEVELS UNDER NORMAL CONDITIONS

The adrenergic neuron stores norepinephrine within vesicles that exhibit characteristic dense cores when examined under the electron microscope after appropriate fixation (60,101). Genesis of a pool of norepinephrine in nerve terminals is ultimately dependent upon biosynthesis of the neurotransmitter. The normal precursor of norepinephrine is tyrosine, usually derived from dietary sources. Three enzymatic reactions within the neuron convert tyrosine to the final product, norepinephrine. The first reaction is the hydroxylation of tyrosine to DOPA (L-3,4-dihydroxyphenylalanine) by tyrosine hydroxylase, which is also the rate limiting step in the biosynthetic pathway (95). The enzyme requires oxygen and a pteridine cofactor (11,74,108). The second enzyme in norepinephrine biosynthesis, aromatic amino acid decarboxylase, is a relatively nonspecific enzyme that catalyzes the decarboxylation of DOPA to dopamine (98). The final reaction, conversion of dopamine to norepinephrine, is catalyzed by dopamine beta hydroxylase, and takes place within or on the storage granules (8,84,93). The rate of norepinephrine synthesis is closely related to the activity of the sympathetic neuron. Thus, both *in vivo* (31,56) and *in vitro* (128,155) sympathetic nerve stimulation are accompanied by increased norepinephrine synthesis from tyrosine, via increased activity of tyrosine hydroxylase. Early studies concluded that this occurred because tyrosine hydroxylase activity was modulated by end-product (norepinephrine) feedback inhibition (74,141,151). Recent evidence

suggests that tyrosine hydroxylase exists in high and low activity forms, with high and low affinity for the pterin cofactor, respectively. Stimulation of adrenergic nerves results in conversion of the enzyme to the high affinity form (155,156). It has been postulated that the conversion to the high affinity form by nerve stimulation may be dependent on a cyclic AMP-dependent protein phosphorylation of either tyrosine hydroxylase or one of its activators or inhibitors (156). Regardless of the mechanism, increased norepinephrine synthesis in response to nerve stimulation is an important mechanism to replenish the stores of norepinephrine released, and maintain constant levels of norepinephrine in the nerve terminal (6).

Catabolism of norepinephrine is initiated through two major metabolic pathways: oxidative deamination by monoamine oxidase (MAO) and methylation by catechol O-methyltransferase (COMT) (134). Monoamine oxidase is primarily localized in mitochondria (28,132), is present in adrenergic neurons (137), and is responsible for catabolism of intraneuronal, cytoplasmic norepinephrine. Treatment of animals with the monoamine oxidase inhibitor, pargyline, leads to increased norepinephrine levels in adrenergically innervated tissues (141). This evidence implicates intraneuronal degradation in modulation of tissue norepinephrine levels. Catechol O-methyltransferase is probably not important for catabolism of intraneuronal norepinephrine. It is more important for catabolism of circulating norepinephrine, and is found in highest concentrations in the liver and kidney (4).

An active neuronal uptake process (Uptake 1) recovers norepinephrine from the interstitium against a concentration gradient (76). This process is carrier-mediated, requires energy, is temperature-dependent, and can be inhibited by prolonged exposure to anoxia or metabolic poisons such as dinitrophenol or cyanide (76). The presence of extracellular sodium ions is necessary (75,129) as are low concentrations of potassium ions (51,116). Neuronal reuptake can be selectively blocked by a variety of agents, cocaine and desmethylimipramine being two of the most potent antagonists (77). Because Uptake 1 is rapid and efficient (75% to 80% of norepinephrine released from nerve terminals may be recovered) it has been implicated as the process that terminates the effects of sympathetic nerve stimulation by removing the neurotransmitter from the synaptic area (76,77). Another role of Uptake 1 is to conserve norepinephrine within the nerve terminal and contribute to maintenance of constant levels, especially during conditions of high sympathetic nerve activity. Thus, when rats were pretreated with specific Uptake 1 blockers and exposed to cold (to increase sympathetic activity), cardiac catecholamine levels dropped markedly, but were unaltered if Uptake 1 was not inhibited (6).

Although norepinephrine is synthesized within the granule, norepinephrine is also present in the cytoplasm due to its uptake from the extracellular space. The storage of norepinephrine within vesicles (repackaging) is dependent on an active uptake process from the cytoplasm that concentrates norepinephrine against a concentration gradient (78). The process is temperature dependent, requires ATP and

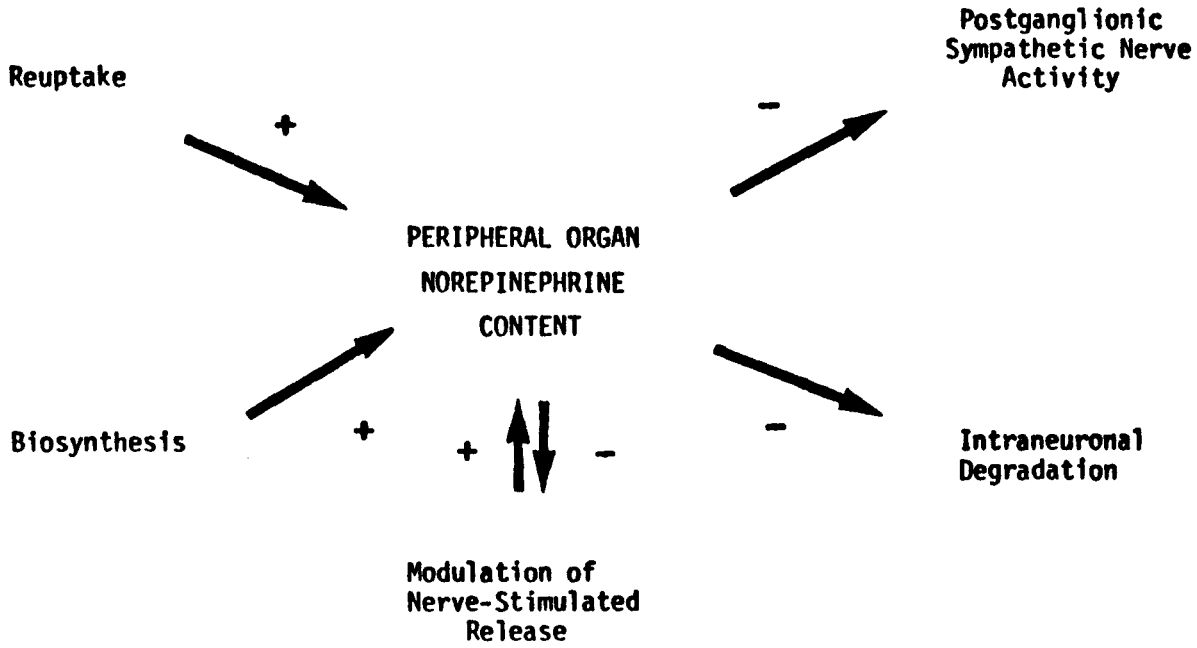
Mg^{++}, and can be selectively inhibited by reserpine (38,106,143). Inhibition of vesicular reuptake results in the degradation of norepinephrine recovered by Uptake 1 in the cytoplasm by the action of monoamine oxidase and lowers norepinephrine content in the terminal (106). Thus, constant norepinephrine levels are dependent on vesicular reuptake as well as Uptake 1.

The amount of norepinephrine released per nerve stimulus can be modulated by numerous factors (92,157). In this regard, many investigations have been directed toward the role of presynaptic receptors. Stimulation of presynaptic alpha adrenergic receptors decreases the amount of norepinephrine overflow measured from various tissues in response to nerve stimulation. Similarly, adenosine, dopamine, and muscarine receptors inhibit nerve-stimulated release of norepinephrine (157). In contrast, stimulation of presynaptic beta adrenergic receptors augments nerve stimulated norepinephrine release (1). Prostaglandins have been reported to have both stimulatory and inhibitory effects related to their series (13). Although manipulation of these processes have not been demonstrated to affect norepinephrine levels in tissues, modulation of norepinephrine release by this mechanism may have effects on norepinephrine pool size under conditions of high sympathetic activity.

Figure 1 summarizes the various processes that determine the norepinephrine content in nerve terminals. Two major processes augment the tissue content of norepinephrine. Biosynthesis of norepinephrine

Figure 1.

Diagram illustrates the processes that determine the norepinephrine content in peripheral tissues. Arrows and + or - indicate the relative contribution of the process on the norepinephrine content (+: increase or -: decrease). See text for details.


Reuptake
+
Postganglionic
Sympathetic Nerve
Activity
−
PERIPHERAL ORGAN
NOREPINEPHRINE
CONTENT
Biosynthesis
+
+
−
−
Intraneuronal
Degradation
Modulation of
Nerve-Stimulated
Release

from its precursor, tyrosine, is the initial process in creating a pool of norepinephrine in the nerve terminal and contributes to maintaining the pool by increases in tyrosine hydroxylase activity and hence synthesis in the face of increased sympathetic drive. Uptake 1 is responsible for conservation of released norepinephrine. The reuptake process is important not only in augmenting the norepinephrine pool, but also is the primary mechanism for terminating the action of norepinephrine at the neuroeffector site. Two major processes decrease the content of norepinephrine in nerve endings: intraneuronal degradation by the action of monoamine oxidase, and release of norepinephrine from nerve terminals by postganglionic sympathetic nerve activity. Finally, modulation of nerve-stimulated release of norepinephrine can either increase or decrease the amount of neurotransmitter released per nerve stimulus. Normally, a steady-state exists between these five processes to maintain a constant pool of norepinephrine in the tissue.

2. NOREPINEPHRINE DEPLETION DURING HEMORRHAGIC SHOCK

a.) Demonstration of the Phenomenon

The earliest quantitation of norepinephrine depletion from peripheral tissues during hemorrhagic shock was in 1961 by Glaviano and Coleman (52). Twelve unanesthetized rabbits were subjected to hemorrhagic hypotension (arterial pressure of 50 mmHg) for three hours. Compared to the twelve normotensive animals, left ventricular norepinephrine content of hypotensive animals was reduced 85% from a

control of 1.05 ± 0.13 µg/gm to 0.16 ± 0.05 µg/gm ($p<0.001$). Conversely, left ventricular epinephrine in the same animals was increased 72% in the hypotensive group from a control of 0.14 ± 0.06 µg/gm to 0.24 ± 0.06 µg/gm ($p<0.01$). Injections of graded doses (25 to 500 ug/kg) of norepinephrine in hypotensive rabbits caused elevation of myocardial norepinephrine in a dose-dependent fashion. The increased myocardial epinephrine content was interpreted as uptake of circulating epinephrine rather than de novo synthesis of the bioamine in the myocardium. This is highly likely because: 1) circulating epinephrine is increased during hemorrhagic shock and 2) the enzyme responsible for the conversion of norepinephrine to epinephrine, phenylethanolamine-N-methyltransferase (PNMT), is present primarily in the adrenal medulla (8). In light of catecholamine-induced augmentation of myocardial contraction and metabolism, the authors speculated that the genesis of myocardial failure in hemorrhagic shock may be due to norepinephrine depletion and concomitant lack of norepinephrine release. In the same model, Coleman and Glaviano (23) subsequently demonstrated that three hours of hemorrhagic hypotension caused norepinephrine depletion from brain, liver, and the previously described depletion from the heart and spleen, but not from skeletal muscle. Epinephrine content was significantly increased in heart, spleen, brain, and liver, and not changed in skeletal muscle.

Subsequently, norepinephrine depletion was reported to occur in other hemorrhagic models. Hift and Campos demonstrated that irreversible hemorrhagic shock depleted cardiac norepinephrine in a

canine model (68). Fasted, pentobarbital-anesthetized mongrel dogs were subjected to a modified Wiggers hemorrhagic procedure (67): Dogs were bled rapidly to 40 mmHg arterial pressure, were maintained at this level by withdrawing or infusing small amounts of blood on demand until the "agonal state", and were sacrificed at that time by excision of the heart. Paired time-matched control dogs were subjected to similar pentobarbital anesthesia and blood pressure measurement. Ventricles and atria of shocked dogs contained on the average 50% less norepinephrine than their time-matched controls. In a regional study of cardiac norepinephrine depletion, Gomez-Povina and Canepa (55) reported that the norepinephrine depletion in pentobarbital-anesthetized hemorrhagic dogs was more marked in the left ventricle than in the right ventricle. Dogs were bled to 30 mmHg arterial pressure and maintained for 135 or 180 minutes and then either sacrificed or reinfused with their own blood and maintained for another 300 minutes. The more severe depletion of left ventricular norepinephrine was evident in both the 135 and 180 minute hemorrhage group. The depletion was partially reversed by retransfusion of blood into the dogs. As in the study of Coleman and Glaviano (52), myocardial epinephrine was increased during the hemorrhagic insult.

Farnebo and Hamberger (40) utilized fluorescent microscopy and biochemical techniques to demonstrate depletion of adrenergic nerves in pentobarbital-anesthetized hemorrhagic rats. Rats were bled through femoral arterial catheters to a blood pressure of 35 mmHg, maintained for four hours, and sacrificed. Histochemical determination of norepinephrine demonstrated a decrease of the bioamine in the iris,

spleen, and blood vessel innervation of submandibular and sublingual gland. The heart and parenchymal innervation of the submandibular and sublingual gland were unaffected by hemorrhage. Biochemical determination of norepinephrine demonstrated decreased norepinephrine in brain tissue, marginally decreased norepinephrine in salivary gland, and increased norepinephrine in cardiac tissue. The discrepancy between histofluorescent and biochemical determinations of norepinephrine in the heart were attributed to the interference of quenching phenomenon in the histofluorescent technique. The conclusions were that discrete activation of sympathetic nerves occurred during hemorrhagic shock in the rat, resulting in depletion of norepinephrine from discrete sympathetically innervated organs as well as discrete areas within organs. Recent work by the same group (41) described a more pronounced sympathetic drive in the unanesthetized hemorrhagic rat compared to the anesthetized counterpart. More active sympathetic drive was evidenced by higher plasma catecholamine levels. In the unanesthetized hemorrhagic rat skeletal muscle and adrenal tissue was depleted of catecholamines and cardiac norepinephrine stores were decreased non-significantly.

Neurochemical techniques have localized the norepinephrine depletion to the sympathetic nerve terminal (30,68). In the aforementioned article by Hift and Campos (68) tissues were centrifuged at various speeds to localize the norepinephrine content to specific subcellular fractions. The depletion of norepinephrine was greatest in the particulate fraction that sedimented on high speed centrifugation

(63% in atria; 68% in ventricles) and less in the soluble and coarse fractions. Presumably the particulate fraction was indicative of norepinephrine bound in vesicles at the terminal sites of sympathetic nerves. The report by Hift and Campos (68) was confirmed independently by Dahlstrom and Zetterstrom (30) using a different technique to measure catecholamines. Experiments employed another modified Wiggers procedure: arterial pressure reduced to 35 mmHg, maintained until 40% of the bled volume was reinfused, and followed until death ensued 2 to 4 hours later. Norepinephrine was estimated in the dog spleen by the histochemical fluorescent technique of Falck and Hillarp (39). The fluorescent method allowed visualization of sympathetic postganglionic fibers and their terminal endings as a green fluorescent light. The specificity of the technique was good, but allowed only qualitative assessment of norepinephrine content. Sympathetic nerve terminals of shocked dog spleens were shown to be depleted of norepinephrine. The fluorescence was shown to be associated with what was generally accepted to be adrenergic nerve terminals. Acute denervation of the spleen protected against the depletion of fluorescence during shock. Chronic denervation (4 weeks prior to the experiment) eliminated all norepinephrine fluorescence in the spleen and also protected against gross anatomic abnormalities associated with hemorrhagic shock. Thus, the norepinephrine depletion that occurred during hemorrhagic shock has been shown using different types of measurement to involve depletion of norepinephrine from sympathetic nerve terminals rather than from some other source (i.e. interstitium, cardiac muscle tissue, etc.).

Thus, norepinephrine depletion from various sympathetically innervated organs has been demonstrated in hemorrhagic models of shock. The depletion has been localized to the sympathetic nerve terminals. The difference in the degree of depletion and the number of organs that it encompasses is most readily explained by the different animal species and hemorrhagic protocols that have been studied. Most of the biochemical techniques for catecholamine measurement were variations of the trihydroxyindole fluorescent technique. Because each study used tissues from non-hemorrhaged control animals subjected to the same biochemical technique, the catecholamine analysis is probably not a source of difference for results obtained in the above studies.

b.) Mechanisms of Hemorrhage-induced Norepinephrine Depletion

Other experiments addressed the consequences and mechanisms of norepinephrine depletion during hemorrhagic shock. Extensive investigation of the depletion phenomenon was performed in the laboratories of Fine. Zetterstrom, Palmerio, and Fine (164) subjected adult white rabbits and mongrel dogs to both reversible and irreversible shock. Experiments with rabbits addressd the two types of hemorrhagic shock. Results showed that hearts, livers, and spleens of reversibly shocked rabbits had significantly reduced levels of norepinephrine (42%, 45%, and 60% respectively, compared to controls). The reduction was proportional to the severity of shock. Therefore, irreversible shock caused a greater reduction in cardiac, liver, and spleen norepinephrine content (34%, 36%, and 51% of respectively, compared to controls). Experiments using dogs had similar outcomes. Both hemorrhagic protocols

resulted in decreased norepinephrine content of hearts, livers, and spleens; the depletion was again proportional to the severity of the hemorrhagic insult. Reversible shock reduced cardiac, liver, and spleen norepinephrine to 39%, 84%, and 76% of control, respectively; irreversible shock in dogs further reduced norepinephrine content in the same tissues to 36%, 32%, and 32% of control, respectively. A local anesthetic was applied to the splanchnic nerves and celiac ganglia of an additional group of dogs prior to six hours of hemorrhagic shock. The hearts of these animals (innervated) contained reduced levels of norepinephrine compared to controls; livers and spleens (effectively denervated by the anesthetic) showed no reduction. These data indicated: 1) peripheral tissue norepinephrine content of dogs and rabbits in hemorrhagic shock decreased and 2) blockade of the nervous supply to the liver and spleen of dogs in hemorrhagic shock resulted in normal content of norepinephrine. The authors attributed the norepinephrine-sparing effect of denervation to both a decrease in the stimulus for release and an increase in blood flow to the denervated organs leading to better oxygenation and thus better metabolic function of peripheral nerve endings. Therefore, the depletion was dependent on neural mediation and did not occur in the absence of sympathetic stimulation. Previously, the same group of investigators (114,165) showed that acute denervation (surgical) or blockade (application of dibucaine in oil to the celiac ganglia) of the sympathetic nerves to the splanchnic organs protected against tissue injury and reduced lethality in hemorrhagic shock as well as preventing norepinephrine depletion. They concluded that the release of norepinephrine from the storage sites

was the critical factor in development of the shock syndrome rather than the absolute content of norepinephrine in the nerve terminals of peripheral tissues. The conclusions are similar to their findings during endotoxin shock in the dog (114,164,165).

The report of Farnebo and Hamberger (40) attempted to assess the amount of reuptake and stimulated release of norepinephrine that occurred in tissues isolated from shocked rats. Either a decreased norepinephrine reuptake or an increased norepinephrine release per stimulus is consistent with the depletion of norepinephrine from tissues of animals undergoing high sympathetic drive. Isolated irises (where norepinephrine depletion did not occur) and isolated brain slices (where norepinephrine depletion did occur) were tested. Results indicated that ^{3}H-norepinephrine uptake into irises incubated in vitro from shocked rats was no different from controls. However, brain slices from shocked rats took up significantly less (14%) ^{3}H-norepinephrine than their control counterparts. Release of ^{3}H-norepinephrine from both groups of tissues demonstrated no hemorrhage-induced changes.

c.) Absence of Norepinephrine Depletion during Hemorrhage

It must be noted that norepinephrine depletion has not been shown to occur in all models of hemorrhagic shock. Glaviano and Klouda (53) demonstrated no differences in left ventricular norepinephrine content between alpha chloralose-anesthetized dogs bled to 40 mmHg for four hours and sham-operated time-matched controls. The authors attributed the results to the open-chested preparation and the extensive

surgical procedures performed on both shock and control dogs. They also mentioned the species difference between this and their earlier studies in the rabbit. Harrison, Chidsey, and Braunwald (64) demonstrated no depletion of atrial norepinephrine in chloralose-anesthetized dogs bled to an arterial pressure of 50 mmHg for four hours. However, they demonstrated that the effect of a tyramine-induced pressor response was reduced greater during shock than a norepinephrine-induced pressor response. Radiolabeled norepinephrine release experiments demonstrated that the amount of norepinephrine released by tyramine was reduced during shock even though the norepinephrine content was unchanged. They speculated that the lack of norepinephrine depletion may have been due to a decrease in a small portion of the norepinephrine store releasable by tyramine, with no change in overall tissue content.

Thus, the evidence for depletion of norepinephrine from tissues of animals in hemorrhagic shock is extensive, although in some models, most notably the anesthetized dog, the depletion is equivocal. The results of Farnebo *et al.* (41) indicated that differences occurred in sympathetic drive between the anesthetized and unanesthetized rat hemorrhagic preparation. Additionally, it should be realized that the two preparations in which no norepinephrine depletion was observed (53,64) were the only reports using chloralose anesthesia. Therefore, not only the presence of anesthesia but also the type of anesthesia may be responsible for the differences in norepinephrine depletion that have been observed during hemorrhage. It is also important to realize that

the underlying mechanism responsible for the depletion during hemorrhage has not been fully elucidated and warrants further study.

3. MECHANISMS OF NOREPINEPHRINE DEPLETION IN ENDOTOXIN SHOCK

Considering the numerous reports of sympathetic hyperactivation and norepinephrine depletion during endotoxic shock, there exists a paucity of information concerning the mechanism of depletion. Investigations by Fine and coworkers that have already been reviewed indicated that the depletion of norepinephrine from splanchnic tissues was neurally mediated (114,164,165). Thus, surgical section or anesthetic infiltration of the splanchnic nerves protected against catecholamine depletion. If endotoxin had a direct effect to release catecholamines from neurons without neural input, denervation would not have had such an effect. The following studies have further investigated the depletion phenomenon and are pertinent to the design of the proposed experiments.

Bhagat *et al.* (7) tested atrial strips from endotoxic guinea pigs (2 to 3 mg/kg) for responsiveness to sympathetic stimulation. Generated isometric tension was recorded from isolated, Tyrode-superfused, left atrial strips. Dose-response curves (concentration of drug versus increase in force generated, reported as per cent of maximal response) were generated for norepinephrine and tyramine. A 50% of maximal effective dose (ED50) was calculated for each experiment. The ratio of ED50 for endotoxic preparations to the

ED50 for control preparations was defined as the sensitivity ratio. Thus, a sensitivity ratio of greater than 1.00 indicated decreased sensitivity of endotoxic preparations compared to controls; a ratio less than 1.00 indicated increased sensitivity of endotoxic preparations compared to controls. Endotoxic pigs were killed from one to forty-two hours after endotoxin. Atrial tissue from endotoxic guinea pigs was less sensitive to norepinephrine than tissue from control animals. The sensitivity ratio had increased by one hour after endotoxin (1.91); lowest sensitivity to norepinephrine was measured at 18 hours after endotoxin (sensitivity ratio of 4.00). By 42 hours after endotoxin challenge, the sensitivity ratio had returned to control levels (1.05). Results were shown for response to tyramine in preparations from control and one hour post-endotoxin guinea pigs. The sensitivity to tyramine was also decreased in the endotoxic tissue (sensitivity ratio: 1.8). The authors hypothesized that increased neuronal reuptake may have been responsible for the results in the endotoxic tissue. Increased reuptake would inactivate the effect of exogenous catecholamines (decreased sensitivity to norepinephrine) as well as locally released norepinephrine (decreased sensitivity to tyramine). This hypothesis was tested by incubating atria from control and endotoxic guinea pigs with ^{3}H-norepinephrine for 30 minutes. Tissues were then rinsed and analyzed for ^{3}H-norepinephrine. Results showed that labeled norepinephrine was actually reduced in atria from endotoxic guinea pigs (760 $\pm$ 25 dpm/mg; mean $\pm$ S.E.M.) compared to controls (1150 $\pm$ 43 dpm/mg). Dose-response curves for norepinephrine were also generated after cocaine treatment. No differences between groups of tissues were reported: Cocaine

increased the sensitivity to norepinephrine by 18-fold in control and 15-fold in endotoxic preparations. Situations in which decreased reuptake of norepinephrine occur should result in decreased sensitivity to cocaine. No statistical analysis was made (factoral analysis of variance would have been appropriate) to test the differences between 18- and 15-fold sensitization to norepinephrine after cocaine. Additional studies were conducted that demonstrated similar length-tension relationships between control and endotoxic preparations. Thus, decreased sensitivity to norepinephrine and tyramine were not ascribed to differences in physical characteristics between the two groups. The conclusions were that endotoxin pretreatment: 1) decreased atrial sensitivity to norepinephrine, 2) did not affect release (or at least the tyramine-releasable fraction) of norepinephrine because the sensitivity ratio was similar for norepinephrine and tyramine, and 3) did not affect uptake of norepinephrine into nerve terminals because of similar sensitivity to cocaine in both groups. Because of reduced ^{3}H-norepinephrine accumulation in endotoxic tissues and similar effects of cocaine in both groups, it was speculated that a reserpine-like effect may have occurred in endotoxic preparations: norepinephrine was transported as usual into nerve terminals, but was then metabolized rather than stored intact as norepinephrine. This hypothesis is possible, but the method used for norepinephrine isolation (elution from acid-washed alumina columns) did not exclude the monoamine oxidase end-product DOPEG (3,4-dihydroxyphenylglycol) from the material that was counted for radioactivity. Therefore, even if norepinephrine was metabolized within the nerve terminal by monoamine oxidase, the DOPEG as

well as the norepinephrine would have been measured, resulting in similar amounts of tritium recovered in both control and endotoxic preparations. Although no measurements of atrial norepinephrine content were made, the study investigated norepinephrine reuptake, depression of which is a possible mechanism for norepinephrine depletion. In fact, Bhagat and Friedman concluded in an earlier paper (6) that under normal conditions, reuptake may not play a significant role in maintenance of cardiac catecholamine levels; constant levels were dependent on synthesis. However, under conditions of high sympathetic nerve activity, reuptake was important in supplementing synthesis to maintain constant norepinephrine levels.

Additional studies have investigated the process of norepinephrine reuptake during endotoxicosis in various animal models (120,123). Pohorecky et al. (120) in the study that documented norepinephrine depletion, assessed norepinephrine uptake and metabolism in endotoxic rats. Animals were injected intravenously with ^{3}H-norepinephrine one hour after intraperitoneal endotoxin. Rats were killed ten minutes, one, and four hours later. Spleens, hearts, and adrenals were analyzed for ^{3}H-norepinephrine. The method for analysis was similar to that of Bhagat et al. (7), and did not exclude deaminated metabolites of norepinephrine. Results indicated in all three tissues examined that labeled catechols were present in higher concentrations in tissues from endotoxic rats compared to controls. By four hours after ^{3}H-norepinephrine injection the groups were reversed and tissues from control rats contained greater labeled catechols compared to

tissues from the endotoxic group. The results were interpreted to indicate that endotoxin increased the uptake and accelerated the turnover of the catecholamine. Increased norepinephrine reuptake is not consistent with norepinephrine depletion from tissues. However, the measurement was made early after endotoxin (one hour), prior to the time when norepinephrine depletion was observed in the model. It has been proposed that norepinephrine reuptake increases during periods of high sympathetic activity (17,50) to conserve the greater amounts of norepinephrine that are liberated. It is evident from an earlier discussion that plasma catecholamine levels are increased after endotoxin administration, although they were not measured in the present study. Increased concentrations of circulating catecholamines may be a partial explanation for the increased reuptake measured.

Rao et al. (123) studied the effects of endotoxin on hemodynamics and norepinephrine metabolism in the dog. ^{3}H-norepinephrine was administered to dogs 30 minutes before endotoxin (2 mg/kg) injection. Blood samples drawn from the femoral artery were collected 5 minutes before and 3, 5, and 30 minutes after endotoxin administration and analyzed for ^{3}H-norepinephrine. Two hours after endotoxin, spleens, ventricles, left atria, right atria, adrenals, and kidneys were analyzed for ^{3}H-norepinephrine and ^{3}H-normetanephrine. ^{3}H-norepinephrine was measured by a similar technique to Bhagat et al. (7) and did not exclude deaminated metabolites. However, aliquots of the sample were analyzed for the catechol-o-methyl transferase (COMT) end-product, normetanephrine, by a further purification step. Two hours

after endotoxin ^{3}H-norepinephrine was increased in spleens and adrenals, but remained unchanged in the other organs sampled. The data were interpreted to indicate that norepinephrine reuptake was increased in the endotoxic dog. However, neuronal reuptake of tracer doses of norepinephrine occurs rapidly (within 10 minutes). In the experiment, the time lapse between ^{3}H-norepinephrine injection and tissue sampling was extremely long (150 minutes). Furthermore, endotoxin was injected long after norepinephrine reuptake was complete. The results may be more indicative of endotoxin modulation of catecholamine metabolism after reuptake. In all tissues ^{3}H-normetanephrine was increased, suggesting that metabolism of norepinephrine was accelerated during endotoxicosis. However, normetanephrine is formed by an enzyme present only outside the sympathetic nerve terminal. Thus, the increased catabolism is indicative of extraneuronal metabolism. Finally, ^{3}H-norepinephrine was decreased compared to control in plasma of endotoxic dogs at all times sampled; ^{3}H-normetanephrine was increased above control in the endotoxic dog at all times tested. These results were also interpeted to indicate that an increased release and metabolism of norepinephrine took place in the endotoxic dog.

Norepinephrine depletion may occur more readily if release is increased. A modulatory effect on the presynaptic terminal to release more norepinephrine per nerve impulse may be partially responsible for depletion of norepinephrine from nerve terminals. Studies (115,117,118) using electrophysiological techniques investigated <u>in vitro</u> effects of endotoxin to alter presynaptic and postsynaptic events. Experiments

performed on neuromuscular preparations of crayfish indicated that *in vitro* addition of endotoxin increased the frequency of miniature excitatory postsynaptic potentials, decreased facilitation, and increased the evoked excitatory postsynaptic potential without changing membrane resistance (115). The results were consistent with the hypothesis that endotoxin acted on the presynaptic nerve terminal to increase the amount of transmitter substance released in response to an applied stimulus. Electrophysiological experiments that tested the effect of either protein-contaminated endotoxin (crude extract) (117) or RNA-free lipopolysaccharide (purified extract) (118) on frog neuromuscular junctions have been performed. Addition of the crude extract to the *in vitro* preparation first induced an increase in spontaneous miniature endplate potential frequency followed by a decreased frequency with no change in miniature endplate potential amplitude. The effect was calcium sensitive and suggested that endotoxin altered calcium conductance channels in the presynaptic terminal membrane. Use of the purified extract depressed spontaneous and evoked transmitter release in the same model. These studies demonstrated that endotoxin had direct neurotoxic effects. A similar modulation of spontaneous or nerve-stimulated release may have occurred during endotoxicosis in the rat to accelerate norepinephrine turnovers and increase sympathetic stimulation to neuroeffector sites. Thus, local effects of endotoxin or its mediators on peripheral neurons may affect spontaneous or evoked norepinephrine release. These hypotheses are consistent with findings from Fine and associates (114,164,165) that demonstrated the protective effect of denervation on peripheral tissue

norepinephrine depletion during endotoxicosis. Although endotoxin may have an effect to increase nerve-stimulated release of norepinephrine, the effect would not be functionally evident if the nervous supply was cut and no nerve activity was present.

As can be surmised, relatively little information is present in the literature that addresses mechanisms for norepinephrine depletion during endotoxicosis. This is surprising in light of the protective effect that blunting the sympathetic response has to pathologic changes and lethality associated with endotoxic shock.

CHAPTER III

RATIONALE AND SPECIFIC AIMS

Two general themes regarding sympathetic nervous system function during endotoxic shock are evident after review of the related literature. One is that the sympathetic nervous system is activated, indeed, over-activated during endotoxicosis. A second is that the massive, generalized, sympathetic tone is detrimental to the organism in endotoxic shock. Thus, extensive work has implicated the sympathetic nervous system in the pathogenesis of endotoxic shock. The present experiments focus on one manifestation of sympathetic activity during endotoxicosis: norepinephrine depletion from peripheral nerve terminals. Experiments of Fine and colleagues (114,164,165) demonstrated that norepinephrine depletion was avoided if the sympathetic nerve supply to an organ was interrupted before either endotoxin or hemorrhage. Furthermore, splanchnic denervation was associated with reduced histopathologic changes in the splanchnic organs during shock as well as a reduced overall lethality. These types of experiments demonstrated that the depletion was important because it represented the transfer of excessive amounts of norepinephrine from storage sites in the nerve terminal to active sites accessible to receptors in the periphery. Thus, characterization of norepinephrine depletion and elucidation of the mechanisms causing it are essential

steps toward reversal or prevention of the functional hyperactivity of the sympathetic nervous system associated with endotoxic shock.

From the discussion of physiological maintenance of tissue norepinephrine levels (as summarized in Figure 1) several points become apparent. First, for a massive decrease in norepinephrine levels to occur the normal relationship between the five listed processes must change or an additional "depleting" process (such as a direct effect of endotoxin to release norepinephrine) must occur. Second, if a change in an existing process occurred, the net effect would be an increase in one or more processes that decrease content (sympathetic drive, intraneuronal degradation) and/or a decrease in one or more processes that increase content (biosynthesis, neuronal reuptake). Third, from the conclusions of denervation experiments the depletion should be dependent on mechanisms involved subsequent to nerve stimulation, because denervation eliminated the depletion. An additional conclusion is that endotoxin does not act directly on sympathetic nerve endings to increase their permeability and cause release of norepinephrine in the absence of nerve stimulation. This would be an example of an additional "depleting" process mentioned above.

What mechanisms that may cause depletion of norepinephrine are consistent with the findings of the existing literature? Increased release of norepinephrine is a possible consequence of endotoxicosis and may occur because of two effects: increased sympathetic nerve activity and/or increased release of transmitter per nerve stimulus. Increased

release is consistent with the elevation of plasma catecholamine levels and depression of tissue catecholamines during endotoxic shock. Increased sympathetic activity and release of catecholamines is also consistent with baroreceptor activation that would be expected during the hypotensive periods after endotoxin administration.

Another hypothesis for norepinephrine depletion which is consistent with the sympathetic neural manifestations of endotoxicosis is depression of neuronal reuptake of norepinephrine. Decreased recovery of released norepinephrine would lead to increased circulating levels of the catecholamine. Because reuptake conserves much of the released norepinephrine, depression of the process sensitizes the nerve terminals to depletion of the neurotransmitter. Because the depletion associated with decreased reuptake is dependent on release of the transmitter, blockade of the nervous supply to the tissue would also protect against the depletion.

Increased intraneuronal degradation is also a possibility consistent with norepinephrine depletion. This could occur by either an increased activity or amount of monoamine oxidase or by a reduction of the intraneuronal vesicular reuptake process. However, the singular effect of increased degradation is not consistent with some of the neural manifestations of endotoxicosis. No increased circulating catecholamine levels would be expected. In fact, a decrease may occur because of an overall decrease in release subsequent to depressed tissue levels. The mechanism of singular depression of catecholamine

biosynthesis is also consistent with tissue depletion of norepinephrine and inconsistent with the elevated circulating catecholamines of endotoxicosis. Again, depressed synthesis would be expected to decrease release subsequent to depressed tissue levels.

Thus, the major candidates for norepinephrine depletion during endotoxicosis are increased release (by increased sympathetic activity or modulation of nerve-stimulated release) and decreased neuronal reuptake of norepinephrine. Minor or contributory candidates that may synergize with the major proposed mechanisms are depressed biosynthesis and increased intraneuronal degradation.

In light of the existing literature on the sympathetic nervous system during endotoxic shock and subsequent interpretations of the studies, the following specific aims are presented for design of the present experiments:

1. Demonstrate norepinephrine depletion in an endotoxic rat model and further characterize the conditions under which it occurs. The spleen and heart will be used for the following reasons: Both organs contain dense sympathetic innervation and relatively high norepinephrine content and are therefore appropriate for depletion studies. Both organs have been used previously in similar investigations and their further study would facilitate interpretations in light of the existing literature. The heart and spleen are both circulatory organs - the heart is representative of

pumping processes with most innervation being directly to myocardial cells. The spleen is representative of the vascular or resistance compartment with most of its innervation on vascular tissue rather than parenchymal or structural cells.

2. Assess sympathetic activity in the endotoxic model by a more reliable and direct method than previously used. The method of choice is norepinephrine turnover measurement in hearts and spleens by the ^{3}H-norepinephrine decay technique. This procedure reliably reflects sympathetic activity to the organ in question (12,26,27). Additionally, it quantitates sympathetic activity by a non-invasive method in an unanesthetized animal, thus eliminating two of the problems of earlier studies. Analysis of norepinephrine turnovers also reveals semi-quantitative data concerning norepinephrine synthesis during the period of turnover measurement.

3. Measure neuronal reuptake of norepinephrine in hearts and spleens of endotoxic animals that display norepinephrine depletion. Use of radiolabeled norepinephrine either injected into the circulation or incubated with tissues *in vitro* will allow tracing the amount of norepinephrine taken up by tissues. It is important to perform both *in vitro* as well as *in vivo* studies because reuptake after norepinephrine injection is dependent on circulatory distribution of the bioamine. Distribution of blood flow has been shown to change dramatically in the endotoxic state (42,83,159,160). From the *in vitro* studies some additional qualitative information may be

obtained regarding intraneuronal degradation of norepinephrine by assessing the amount of radiolabeled norepinephrine retained in its unmetabolized state.

The aims of the present research are founded on a logical extension of the existing literature and are designed to answer important questions regarding sympathetic function during endotoxic shock. Execution of the experiments should enhance and expand the understanding of the mechanisms of norepinephrine depletion as well as promote the development of interventions to reduce sympathetic hyperactivity in endotoxic shock and its clinical counterparts, sepsis and septic shock.

CHAPTER IV

METHODS

A. Animals and Care

Male Holtzman rats of 300-350 gm body weight were used throughout these studies. Prior to experimentation all rats were maintained in the animal research facility for a minimum of 7 days after receipt from the supplier (Holtzman Company, Madison, Wisconsin) to: 1) adjust to a 12 hr light-dark cycle (light from 0700 hr to 1900 hr, central daylight time), and 2) recover from the stress of shipping. Rats were housed four per cage (cage dimensions: 16" length, 9.5" width, and 7" depth) except where noted to be housed individually (cage dimensions: 7" length, 9.5" width, and 7" depth). Ambient temperature was approximately 72° F and relative humidity was 30% to 50%. Animals were maintained on Purina rat chow and tap water ad libitum. Fasting was induced by removing food but not water at 4 to 6 pm on the day preceding the experiment. Cages were of a hanging variety with wire mesh floors so that rats did not have access to their own feces during the evening of the fast. Thus, rats were post-absorptive by the start of experiments on the following morning.

B. Shock Model

Endotoxins derived from the outer cell walls of gram-negative bacteria have been widely used experimentally to model septic shock. The present experiments employed a simple, reproducible model of endotoxic shock in the rat. The biological activity of an endotoxin isolated from the cell walls of gram-negative bacteria depends upon the cell wall constituents associated with it. The preparation of endotoxin used in these experiments was Salmonella enteritidis endotoxin prepared by the Boivin method (extraction by trichloroacetic acid). The toxin thus obtained was primarily lipopolysaccharide in complex with protein from the cell wall. Endotoxin was purchased from Difco Laboratories, Detroit, Michigan. The endotoxin was prepared daily in 0.9% saline. Fasted rats under light ether anesthesia were injected with endotoxin (5 mg/rat or about 14 to 17 mg/kg, except where noted) intravenously via the dorsal vein of the penis. All injections were performed between 0700 hr and 1000 hr to minimize the effects of diurnal sensitivity to endotoxin. This regimen induced a 60% to 80% lethality at 24 hours (seasonal variation in lethality occurred). Each batch of endotoxin was checked for potency prior to experiments. Control rats under ether anesthesia were injected intravenously with 0.9% saline. The rats were returned to their cages (allowed access to water but not food) and followed until just prior to death (evidenced by loss of the righting reflex) if it occurred by 5 hours post-endotoxin; rats still alive 5 hours post-injection were sacrificed at that time.

The shock state progresses at different rates in individual animals. Use of the present protocol allowed study of both mildly and severely shocked rats. Because glucoregulatory dysfunction of endotoxic shock results in a progressive decline of plasma glucose after an initial hyperglycemia (45), the plasma glucose concentration was used as an indicator of the severity of the shock state. Mild or early phase shock is indicated by a plasma glucose greater than 40 mg/dl; severe or late phase shock is indicated by a plasma glucose less than 40 mg/dl. All endotoxic rats were, therefore, divided into mild or severe shock on the basis of their plasma glucose levels at the time of sacrifice.

C. Plasma Glucose Measurement

Rats were sacrificed by decapitation with a guillotine (Stoetler Apparatus Company). Mixed arterial and venous blood samples were collected from the neck wound in 250 µl heparin-treated microfuge tubes. The blood was stored on ice for short periods (5 to 30 minutes) before centrifugation in a Beckman Model 152 microfuge. Samples were then frozen until glucose analysis was performed (usually within one week).

Plasma glucose was measured on a Yellow Springs Instrument Model 23A Glucose Analyzer. The instrument utilizes an oxidase enzyme hydrogen peroxide probe developed by Dr. Leland Clark. The principles on which the analyzer operates are as follows: D-Glucose and oxygen are converted to gluconic acid and hydrogen peroxide via the enzyme glucose

oxidase (reaction 1). The hydrogen peroxide is then oxidized by a platinum electrode (the anode) to liberate hydrogen ions, oxygen, and electrons (reaction 2). A silver cathode then reduces the oxygen and hydrogen ions to water (reaction 3). The oxidation of hydrogen peroxide creates a current that is proportional to the amount of glucose in the sample.

In practice, the glucose probe is the platinum anode covered by a two-layered membrane. The outer membrane is composed of a polycarbonate material that will allow diffusion of small molecules but will exclude enzymes. The inner membrane is composed of a cellulose acetate material that will pass hydrogen peroxide, oxygen, water, and salts, but will exclude glucose, ascorbic acid and other substances that may interfere with the analysis. The glucose oxidase is immobilized between the membranes in a thin layer of resinous material. Thus, glucose in the sample diffuses through the outer membrane, is oxidized to hydrogen peroxide by the glucose oxidase, and then diffuses through the inner membrane for the reaction at the platinum electrode. The buffer in which the glucose is diluted contains a catalase that destroys any hydrogen peroxide that diffuses back into the sample chamber. The electrode system acts as a polarograph. The platinum electrode is polarized at 0.7 volts with respect to the cathode to maximize the specificity of reaction 3 for oxidation of hydrogen peroxide. The polarization voltage and the inner membrane effectively filter out all interfering substances. The current necessary to maintain a constant voltage (0.7 volts) across the electrodes is then proportional to the

amount of hydrogen peroxide oxidized, and hence the amount of glucose present in the sample. Therefore, the glucose measurement is dependent on the amount of glucose in the sample, and not on its concentration. For accurate measurements it is critical to maintain constant sample size. This method has been shown to give comparable results as other methods (72). The instrument was routinely calibrated against known standards and checked for linearity before sample analysis. All samples were analyzed in duplicate, and an average of the results was calculated for the final glucose concentration.

D. Tissue Catecholamine Measurement

Tissue levels of catecholamines (norepinephrine and epinephrine) were estimated by the trihydroxyindole fluorometric assay according to Crout (29). According to this method, the catecholamines are purified by the acid-washed alumina method first used by Shaw (135) and later modified by Lund (100). The fluorometric analysis has been adapted to the Autoanalyzer by Jellinek *et al.* (79). The following section includes a description of these procedures as well as an illustration of typical fluorometric data.

1. TISSUE SAMPLING AND STORAGE

After decapitation, hearts and spleens were rapidly removed and blotted to remove excess blood. Tissues were frozen rapidly by

compression between a pair of Wollenberger tongs precooled in liquid nitrogen and immediately immersed in liquid nitrogen to accelerate the freezing process. Spleens were frozen intact, without removal of the capsule. Hearts were frozen with only the ventricles compressed between the faces of the tongs. The atria were cut away before immersion in liquid nitrogen. Thus, all cardiac catecholamine levels reflect average ventricular (left and right) content of the bioamines. The total time to remove and freeze the samples was about two minutes from the time of decapitation. Frozen tissues were subsequently stored in liquid nitrogen until analyzed (typically one to two weeks). A timed storage study in this laboratory demonstrated that degradation of catecholamines during storage time was negligible.

2. CATECHOLAMINE PURIFICATION

Tissues were weighed frozen on a Roller-Smith balance and placed in 10 ml of 10% trichloroacetic acid. As tissues thawed, they were homogenized with a Brinkman Kinematica Polytron for 20 seconds at full speed. The homogenate was filtered on fluted Sharkskin filter paper; the precipitated protein was discarded.

Five ml of the filtrate was transferred to a 50 ml Erlenmeyer flask containing the following: 1) 10.0 ml of cold 0.2 M sodium acetate (NaOAc), 2) 0.5 ml of cold 0.2 M ethylenediaminetetraacetate (EDTA), and 3) 1 drop of 1% phenolphthalein. The solution was titrated with cold 0.5 N NaOH to pH 8.4, indicated by a pale pink color. The solution was

poured onto a glass column containing 0.5 gms of acid-washed alumina. Columns were custom-made and were composed of a 50 ml reservoir at the top, below which was a 15 cm column (I.D. 0.5 cm). At the lower end of the column a constriction allowed placement of glass wool that acted as a trap for the alumina. Flow rate was typically one to two ml/min. At pH 8.4 good specificity was obtained for adsorption of the negatively charged catecholamines onto the positively charged alumina. Ten ml of cold distilled water were added to the column as a rinse. Finally, the catecholamines were eluted from the column by addition of 5 ml of 0.2 M acetic acid. The eluates were sealed with parafilm and stored at 5° C until analyzed (within one to two weeks). The catecholamines have been shown in this laboratory to be stable over this period. Recovery of norepinephrine from the columns was routinely 80%. Values were reported as uncorrected for the per cent recovery.

3. CATECHOLAMINE ANALYSIS

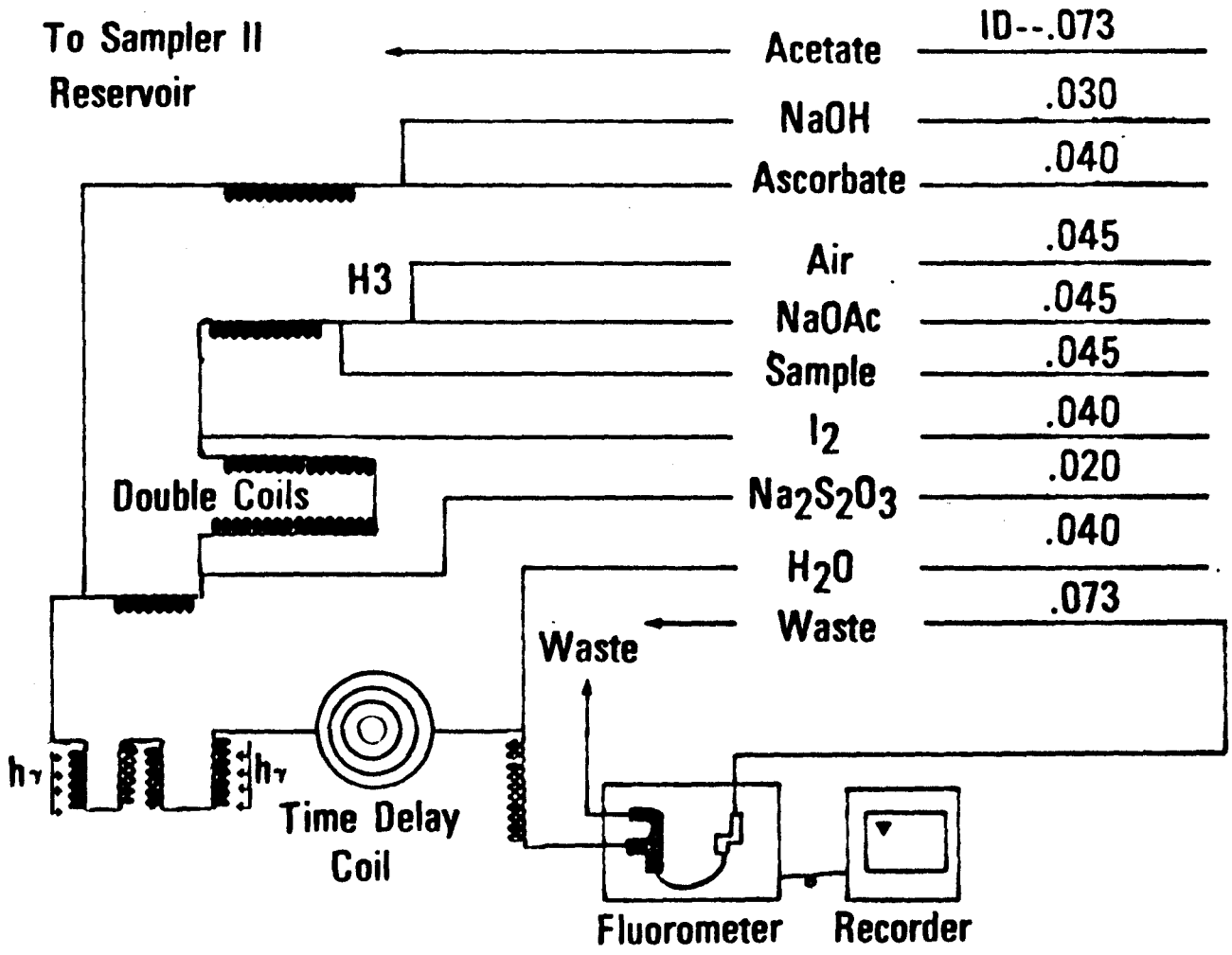
After mixing with slightly acidic sodium acetate buffer (NaOAc + EDTA), the eluate was analyzed for catecholamines by oxidation of the norepinephrine and epinephrine to their respective 2-iodo aminochrome derivatives with iodine. Subsequent treatment with strong NaOH (30%) caused tautomerization of the aminochromes to fluorescent derivatives of 3,5,6-trihydroxyindole. Because the indoles were unstable in the presence of oxygen, ascorbic acid was added to remove dissolved oxygen from the system. The flow diagram for adaptation of this procedure to an automated process on the Technicon Autoanalyzer is illustrated in

Figure 2. Concentrations of the reagents were as follows: 0.2 N acetic acid, 30.0% NaOH, 1.0 M NaOAc (either at pH 6.5 or 3.5), 0.1 N I_2, 0.05 N thiosulfate, and 7.5 mM ascorbate. The fluorometer was outfitted with a flow-through cell and interfaced with a Beckman strip chart recorder. The primary fluorometer filter (47B) was of a narrow pass variety at 436 nm. The secondary filter (2A-12) was a sharp cutoff filter above 510 nm.

Differential estimation of norepinephrine and epinephrine was accomplished by carrying out the oxidation to the aminochrome with buffer at pH 3.5 and pH 6.5. At the less acidic pH both bioamines are oxidized to the aminochrome stage; at the more acidic pH only epinephrine is significantly oxidized. In practice, the differentiation of catecholamines was performed by analyzing the sample four times in the following modified conditions: 1) NaOAc at pH 6.5, 2) NaOAc at pH 3.5, 3) NaOAc at pH 6.5 containing 50 ng/ml norepinephrine, and 4) NaOAc at pH 6.5 with the ascorbate and distilled water reagent tubes reversed. Under condition 1, both norepinephrine and epinephrine are oxidized, while under condition 2 only epinephrine is oxidized. The fluorometric peak height in condition 2 is proportional to the epinephrine concentration in the sample; the difference between the peak heights in condition 1 and 2 is proportional to the norepinephrine concentration. Condition 3 is an internal standard that raises the background level of norepinephrine and allows for estimation of fluorescent quench that occurs. Condition 4 introduces the ascorbate into the sample mixture after oxygen has destroyed the 3,5,6-trihydroxyindole derivative of the

Figure 2.

Schematic diagram illustrates the tubing organization for catecholamine analysis on the Autoanalyzer. Numbers along right margin indicate the inner diameter (inches) of tubes that draw the indicated reagents. Because the roller pump rotates at a constant velocity, the tubing diameter regulates the flow.


To Sampler II
Reservoir
Acetate
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NaOH
.030
Ascorbate
.040
Air
.045
H3
NaOAc
.045
Sample
.045
I2
.040
Double Coils
Na2S2O3
.020
H2O
.040
Waste
.073
Waste
hν
hν
Time Delay
Coil
Fluorometer
Recorder
Primary Filter 47B
Secondary Filter 2A-12

catecholamines. This measurement is a blank that yields background fluorescence in the sample. In addition to unknown samples, one known concentration (repeated three additional times) of norepinephrine and epinephrine (referred to as daily standards) as well as a 0.2 M acetic acid sample (repeated three additional times), the vehicle for the catecholamines, was run under the above conditions as standards and blank, respectively. Because the fluorometer output describes a linear relationship to catecholamine concentration, only one blank and one known concentration of norepinephrine and epinephrine are necessary to calculate catecholamine concentrations of the unknown samples.

4. CALCULATION OF NOREPINEPHRINE AND EPINEPHRINE

Norepinephrine (NE) and epinephrine (E) were calculated for each sample by solving the following simultaneous equations:

$$(a/b) \times !NE! + (c/d) \times !E! = \text{peak height of sample at pH 6.5}$$

$$(e/b) \times !NE! + (f/d) \times !E! = \text{peak height of sample at pH 3.5}$$

where:

a = peak height of norepinephrine standard at pH 6.5;

b = concentration of norepinephrine standard;

c = peak height of epinephrine standard at pH 6.5;

d = concentration of epinephrine standard;

e = reading of norepinephrine standard at pH 3.5;

f = reading of epinephrine standard at pH 3.5;

!NE! = concentration of norepinephrine in sample;

!E! = concentration of epinephrine in sample;

Note: All peak heights are previously corrected for quench using the internal standard.

The calculations were performed on a PDP-12 computer (Digital Equipment Co.). The computer program (FOCAL) is shown in Figure 3. The peak heights from the strip chart recorder were entered into the computer using the following code letters:

Standards -

P = NE reading at pH 6.5 NaOAc buffer;

Q = NE reading at pH 3.5 NaOAc buffer;

R = E reading at pH 6.5 NaOAc buffer;

S = E reading at pH 3.5 NaOAc buffer;

Samples -

LABEL = Name of sample;

BLK = Blank reading;

RED = Reading at pH 6.5 NaOAc buffer;

EPI = Reading at pH 3.5 NaOAc buffer;

STD = Reading using internal standard;

WT = Weight of tissue;

An example of the recorder output from the fluorometer for known samples is shown in Figures 4 and 5. Nine different concentrations of norepinephrine and/or epinephrine were made from stock solutions of 10 µg/ml and diluted into 0.2 M acetic acid. No mixture was made from serial dilutions of another. Nine pairs of samples were run. Figure 4

Figure 3.

FOCAL program used to calculate tissue norepinephrine and epinephrine content. Output is in µg of bioamine per gm of tissue wet weight.

```
C-PS/8 FOCAL, 1971

01.10 C THIS PROGRAM CALCULATES THE CONCENTRATION OF CATECHOLAMINE
01.11 C PER WET WEIGHT OF TISSUE SAMPLE.  ASSAY IS ACCORDING TO
01.12 C CROUT, AUTOMATED BY JELLINEK, AND MODIFIED BY S. B. JONES.
01.20 S A=0;S B=0;S C=0;S V=0

02.15 T !!"ENTER DATA FROM DAILY STANDARDS."!!
02.16 T "          NE          NE          EPI         EPI "!
02.17 T "       PH 6.5      PH 3.5      PH 6.5      PH 3.5"!!
02.19 F I=1,4;D 3
02.20 T !,%7.03,"AVE:",A,B,C,V
02.30 G 4.1

03.10 A "          "P,"       "Q,"       "R,"       "S;T !
03.20 S A=A+P/4
03.30 S B=B+Q/4
03.40 S C=C+R/4
03.50 S V=V+S/4

04.10 A !!"HOW MANY EXPERIMENTAL SAMPLES?  "NO
04.15 T !!!"ALL OUTPUT VALUES ARE UG CATECHOLAMINE/GM TISSUE WET WEIGHT."
04.30 T !!!"LABEL",:20,"BLANK",:28,"PH 6.5",:37,"PH 3.5"
04.31 T :46,"INT STD",:56,"WT (GM)",:66,"ERRORS?"!!
04.40 F I=1,NO;D 5
04.50 G 8.10

05.30 A JK,:21,BLK,:29,RED,:38,EPI,:48,STD,:57,WT,:69,ERR
05.32 I (ERR-0Y)5.40,5.20
05.40 S T=RED-BLK;S G=EPI-BLK;S SN=STD-RED+A;S SE=STD-RED+C
05.50 S H=V/SE
05.52 S XJN=10*WT;S CN=((N*T-G)/(N*SN-B))*(XJN/WT)*.05
05.56 S SAT=CN/((XJN/WT)*.05)
05.58 S CE=((G-B*SAT)/V)*(XJN/WT)*.05
05.60 T !,%4.03,:20,"NOREPI:",CN,:42,"EPI:"CE
05.61 T :61,"TOTAL:"CN+CE,!!

08.10 A !!"ANYMORE?"AN
08.12 I (AN-0Y)8.20,8.14
08.14 A !"NEW STANDARDS?"NS
08.16 I (NS-0Y)8.18,2.15
08.18 A !"NEW SAMPLES?"NN
08.19 I (NN-0Y)8.10,4.10
08.20 T !!!"THIS IS THE END OF THE CATECHOLAMINE ASSAY PROGRAM."!
08.21 Q

09.20 T !!"REPEAT SAME SAMPLE."!!;G 5.30
```

Figure 4.

Output from Beckman chart recorder illustrates fluorometric peaks from samples run in buffer at pH 6.5. Concentrations of norepinephrine (NE) and epinephrine (E) are indicated above the peaks. Samples 7-12 contain a mixture of norepinephrine and epinephrine that is a total of 60 ng of catecholamine per ml.

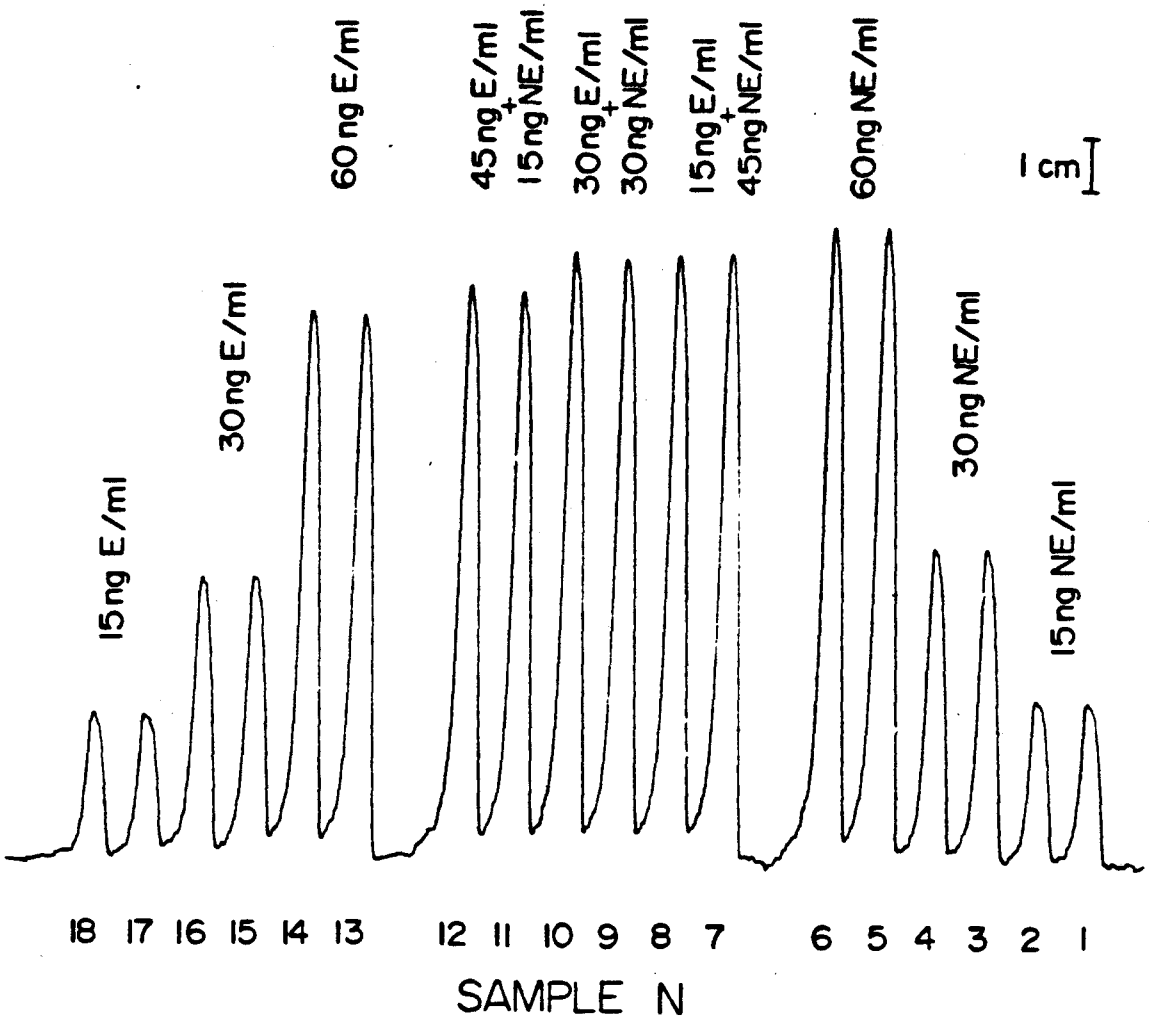
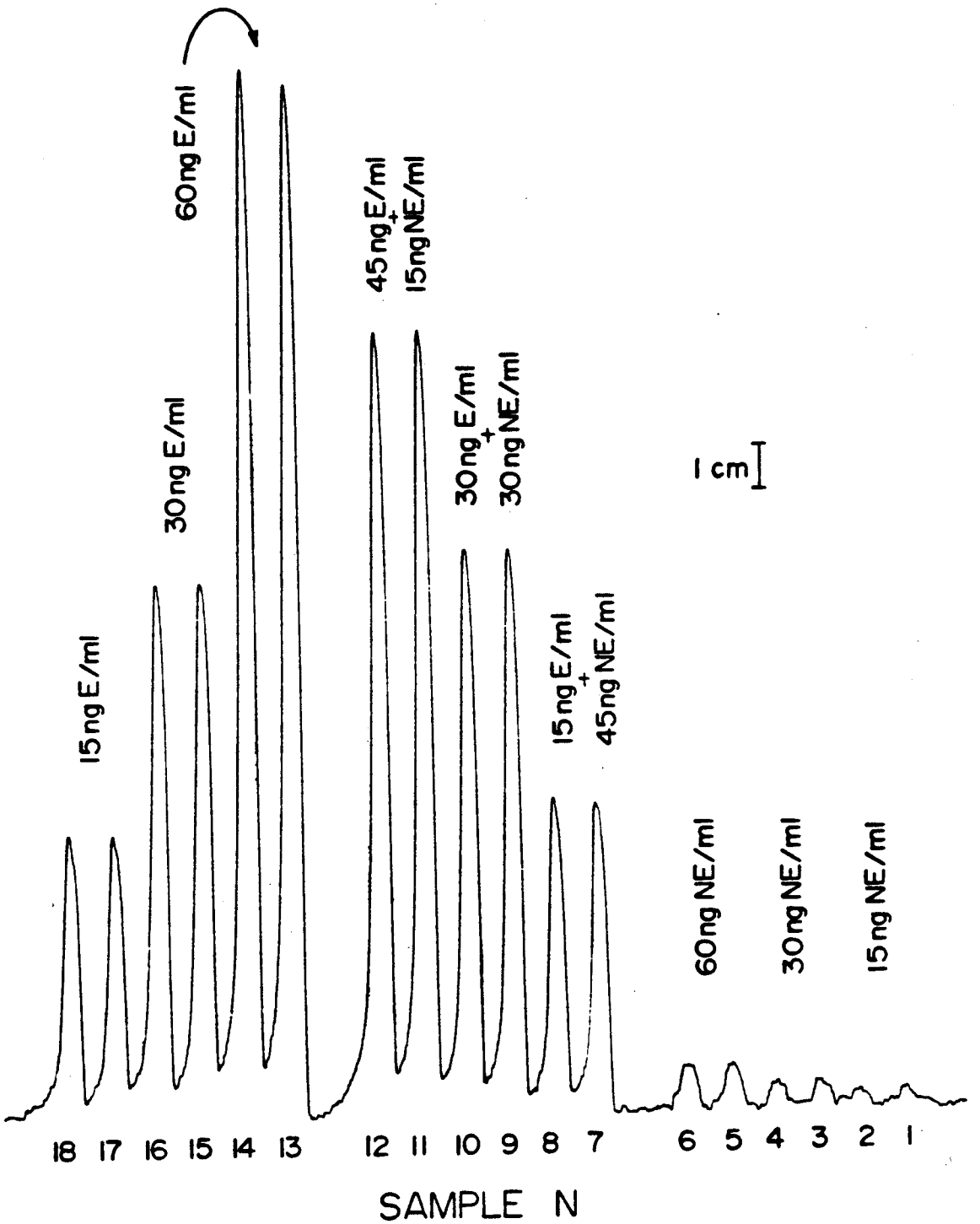

15ng E/ml
30ng E/ml
60 ng E/ml
45ng E/ml + 15ng NE/ml
30ng E/ml + 30ng NE/ml
15ng E/ml + 45ng NE/ml
60ng NE/ml
30ng NE/ml
15ng NE/ml
1 cm
18 17 16 15 14 13 12 11 10 9 8 7 6 5 4 3 2 1
SAMPLE N

Figure 5.

Output from Beckman chart recorder illustrates fluorometric peaks from samples run in buffer at pH 3.5. Concentrations of norepinephrine (NE) and epinephrine (E) are indicated above the peaks. Samples 7-12 contain a mixture of norepinephrine and epinephrine that is a total of 60 ng of catecholamine per ml.


60ng E/ml
30ng E/ml
15ng E/ml
45ng E/ml + 15ng NE/ml
30ng E/ml + 30ng NE/ml
15ng E/ml + 45ng NE/ml
60ng NE/ml
30ng NE/ml
15ng NE/ml
1 cm
18 17 16 15 14 13 12 11 10 9 8 7 6 5 4 3 2 1
SAMPLE N

depicts peak heights for samples run with pH 6.5 buffer; Figure 5 depicts peak heights for samples run with pH 3.5 buffer. Note that samples 5 through 14 contained a total catecholamine concentration of 60 ng/ml (norepinephrine and/or epinephrine). At pH 6.5 both catecholamines were oxidized to approximately the same degree. Therefore, peak heights 5 through 14 were of similar magnitude. However, at pH 3.5 only the epinephrine was significantly oxidized, and thus the peak heights were primarily dependent on the concentration of epinephrine present in the sample.

The linearity of the system is shown graphically in Figures 6 and 7. The peak heights (cm) for the pure epinephrine and norepinephrine knowns (15, 30, and 60 ng/ml) were plotted versus their concentrations (ng/ml). Additional points were the blank (0 ng/ml) and the normal daily standard (50 ng/ml). These two concentrations are the two standards used for calibration of a typical analysis. Normal unknown data values fall within the extremes of the graphs. Figure 6 depicts the results for the samples run in pH 6.5 buffer (data from Figure 4). Note that norepinephrine oxidation, and hence its fluorescence, is marginally greater than for epineprine at this pH. Figure 7 represents the data recorded at pH 3.5 (data from Figure 5). At this pH epinephrine oxidation and fluorescence is approximately twenty times higher than norepinephrine oxidation. Regression lines were calculated from all five data points for each line. The correlation coefficient, r, for each of the four lines was greater than 0.99. The equations for the lines in the form $y = m(x) + b$ are:

Figure 6.

Graph displays the linear response of the fluorometer to epinephrine and norepinephrine analyzed in pH 6.5 buffer. The peak height (cm) is plotted versus the catecholamine concentration (ng/ml) for the raw data of Figure 4. Details of linear regression are in text.

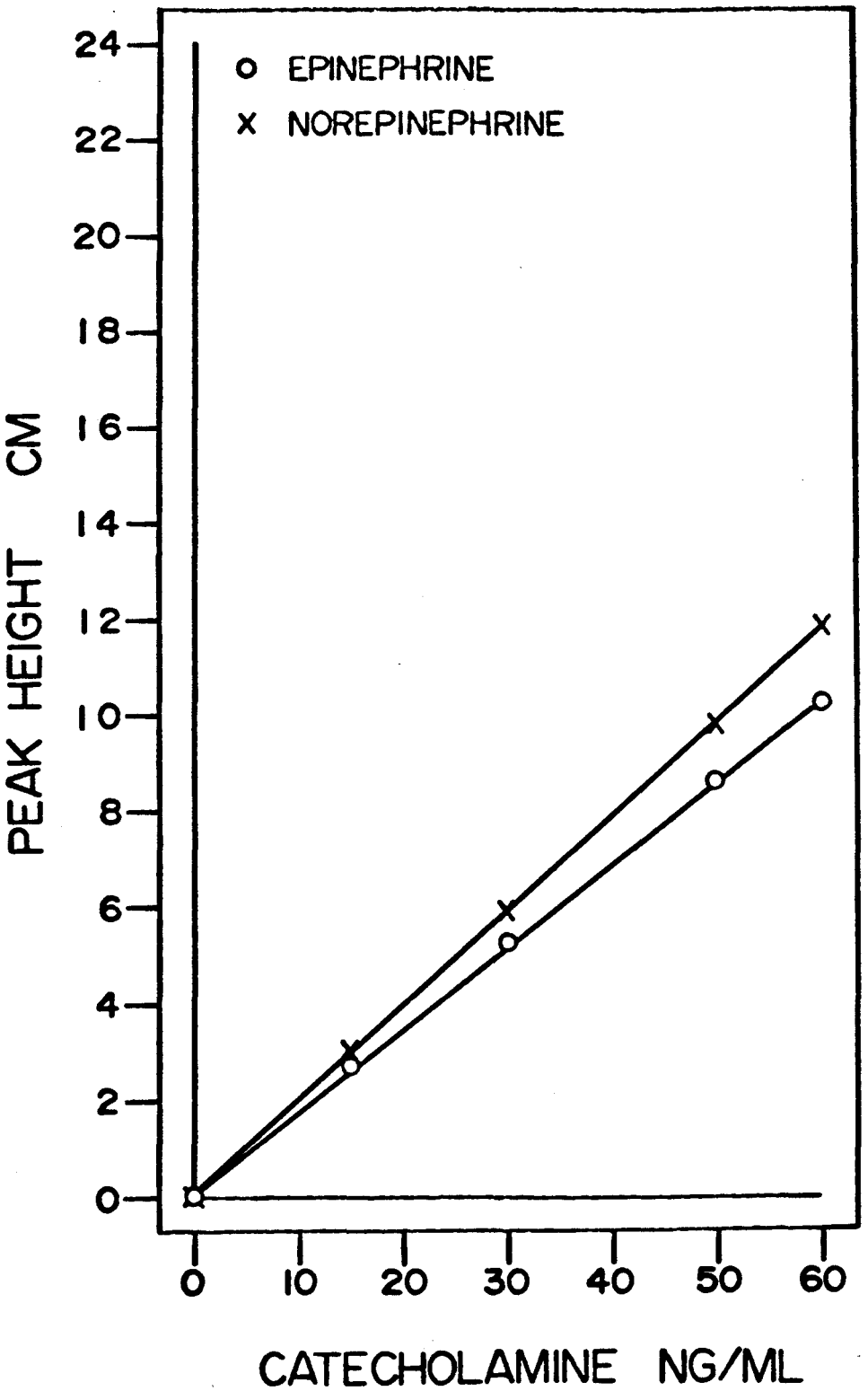
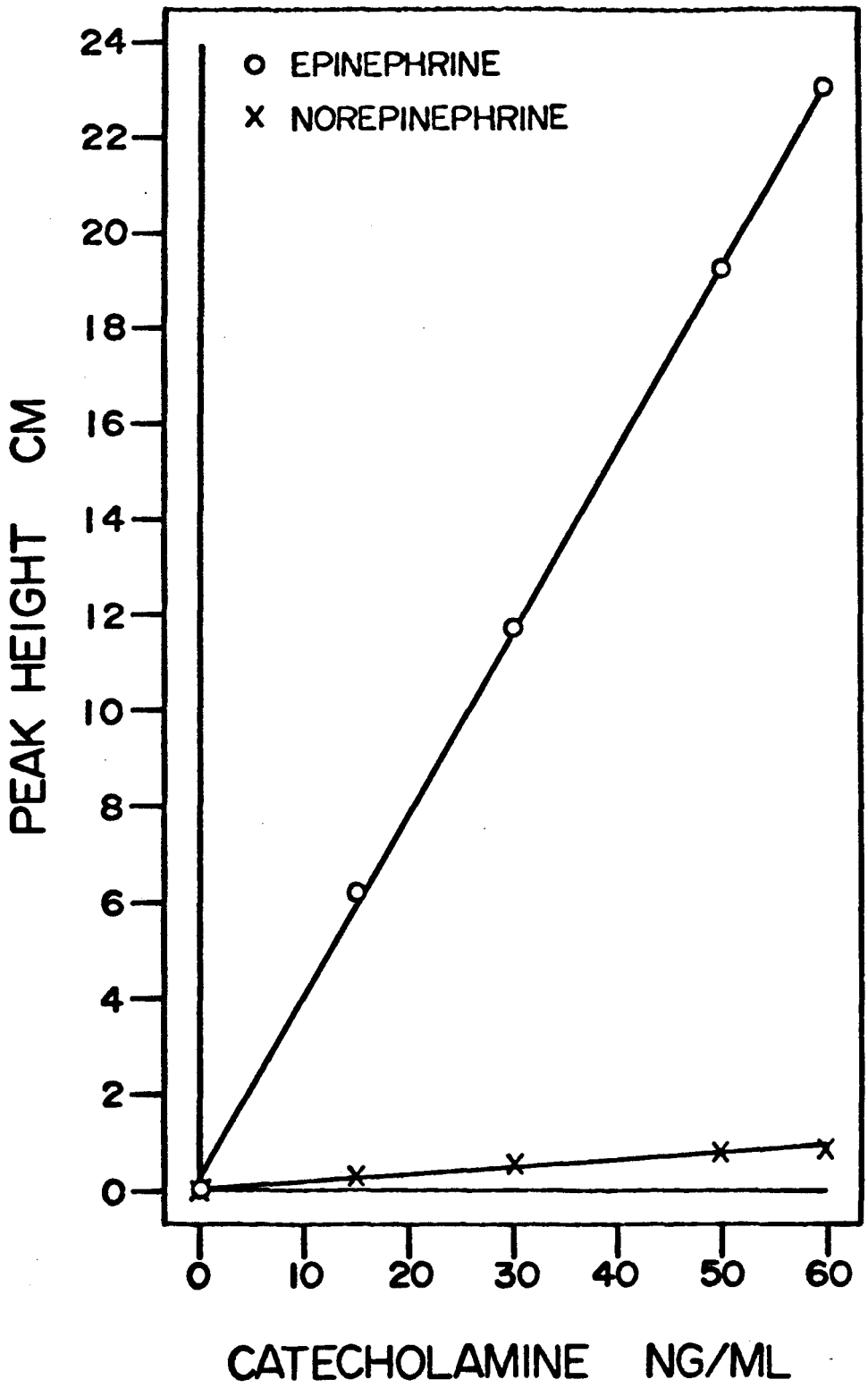

EPINEPHRINE
NOREPINEPHRINE
PEAK HEIGHT CM
24
22
20
18
16
14
12
10
8
6
4
2
0
0
10
20
30
40
50
60
CATECHOLAMINE NG/ML

Figure 7.

Graph displays the linear response of the fluorometer to epinephrine and norepinephrine analyzed in pH 3.5 buffer. The peak height (cm) is plotted versus the catecholamine concentration (ng/ml) for the raw data of Figure 5. Details of linear regression are in text.


EPINEPHRINE
NOREPINEPHRINE
PEAK HEIGHT CM
24
22
20
18
16
14
12
10
8
6
4
2
0
0
10
20
30
40
50
60
CATECHOLAMINE NG/ML

Norepinephrine at pH 6.5: $y = 0.197x + 0.023$,

Epinephrine at pH 6.5: $y = 0.171x + 0.089$,

Norepinephrine at pH 3.5: $y = 0.149x + 0.053$, and

Epinephrine at pH 3.5: $y = 0.381x + 0.223$.

Table I depicts the repeatability and the accuracy of the analysis system. The actual concentrations of norepinephrine and epinephrine for each known sample as well as the calculated values are illustrated. Calculated values were derived from the data of Figures 4 and 5, and the respective internal standard and blank runs (not illustrated). Results were calculated using the previously described FOCAL program with a slight modification to account for absence of tissue weights and to calculate the output as ng/ml. The calculated values are repeatable and extremely close to the actual concentrations.

5. RECOVERY OF CATECHOLAMINES FROM COLUMN CHROMATOGRAPHY

All of the catecholamines from the filtrate are not recovered in the eluate from the alumina chromatography isolation. The amount of recovery is closely related to the preparation of acid-washed alumina and the technique by which the catecholamines are isolated. Typically, the recovery of catecholamines from the column is 80%, and is highly reproducible. The per cent recovery was measured numerous times while the technique was initially learned, and was subsequently performed every time a new batch of acid-washed alumina was prepared. The procedure for checking the per cent recovery is as follows:

TABLE I: ACTUAL AND CALCULATED CONCENTRATIONS

OF NOREPINEPHRINE AND EPINEPHRINE FROM DATA OF FIGURES 4 AND 5

	NOREPINEPHRINE (ng/ml)		EPINEPHRINE (ng/ml)	
Sample No.	Actual	Calculated	Actual	Calculated
1	15	15	0	0
2	15	15	0	0
3	30	30	0	0
4	30	29	0	0
5	60	60	0	0
6	60	61	0	0
7	45	43	15	16
8	45	43	15	16
9	30	29	30	31
10	30	31	30	31
11	15	14	45	45
12	15	17	45	44
13	0	-1	60	60
14	0	-1	60	61
15	0	0	30	31
16	0	0	30	31
17	0	-1	15	16
18	0	-1	15	16

Nine different concentration mixtures (norepinephrine and/or epinephrine) were made in 0.2 M acetic acid. Two samples (1 and 2, 3 and 4, etc.) were taken from each mixture, treated as column eluates, and analyzed as described in the text. Therefore, these data did not rely on serial dilutions of samples. Data from Figures 4 and 5 as well as additional data using an internal standard and blank were used to calculate the values with the FOCAL program.

Several tissues were ground and pooled to yield about 50 ml of filtrate; eight 5 ml samples were removed. Samples 1-4 were processed as described above for catecholamine purification. Samples 5-8 were purified after addition of 250 ng of norepinephrine. The result was an additional 50 ng/ml in the final 5 ml of eluate. The results of a typical per cent recovery are shown on Table II.

E. ^{3}H and ^{3}H-Norepinephrine Analysis

For experiments in which ^{3}H-norepinephrine was used, total ^{3}H or ^{3}H-norepinephrine in tissues was measured as follows: Aliquots (0.5 ml) of the protein-free filtrate (in the case of total ^{3}H measurement) or the alumina eluates (for ^{3}H-norepinephrine measurement) were dissolved in 14.5 ml of PCS scintillation fluor (Amersham). Samples were counted for ^{3}H in an Isocap/300 scintillation counter (Nuclear Chicago).

Quench correction was performed by the standard channels ratio method (153). Use of this technique relies on the effect of quench to decrease the average pulse height of the ^{3}H energy spectrum; the pulse height spectrum will shift toward a lower energy level in a count rate versus pulse height graph. A known standard with no quench and with various amounts of added quench is counted under two conditions: 1) with the window discriminators open only to a ^{3}H channel (A) and 2) with the window discriminators open to a full ^{14}C window (B). The ratio of channel A to channel B counts is dependent on the amount of pulse height

TABLE II. RECOVERY OF NOREPINEPHRINE (NE) FROM COLUMN CHROMATOGRAPHY

(Explanation of protocol is in text.)

Substance	Peak Height	Avg Peak Height
50 ng/ml NE Standard	6.5	
50 ng/ml NE Standard	6.5	6.53
50 ng/ml NE Standard	6.6	
50 ng/ml NE Standard	6.5	
Sample 1	2.9	
Sample 2	2.9	2.88
Sample 3	2.8	
Sample 4	2.9	
Sample 5 + NE	8.2	
Sample 6 + NE	8.5	8.23
Sample 7 + NE	8.2	
Sample 8 + NE	8.0	

where: standard = A (known)

sample = B (unknown)

sample + NE = C (known + unknown)

then: 100 x (C - B)/A = per cent recovery

or: 100 x (8.23 - 2.88)/6.53 = 81.9% recovery.

shift and hence the amount of quench. From the data obtained on known activity standards, a standard curve is constructed plotting per cent counting efficiency versus ratio of channel A to channel B counts per minute. For each experimental sample, the channel count ratio is determined and the per cent counting efficiency is determined from the standard curve. In practice, a FOCAL program has been written to construct a standard curve for each daily run from the commercially purchased quenched standards and to correct each sample for its counting efficiency and convert it from cpm to dpm. The program is illustrated in Figure 8. Minor alterations of the program were made to tailor the output to the particular experiment. The specific program depicted was used for the *in vitro* norepinephrine uptake experiments. Efficiency for counting ^{3}H in this system was typically 40-45%. All tritium data were expressed as dpm per unit of tissue wet weight.

F. Norepinephrine Reuptake Measurement

1. IN VIVO REUPTAKE MEASUREMENT

Norepinephrine reuptake was assessed *in vivo* by measuring the amount of intravenously injected ^{3}H-norepinephrine retained by hearts and spleens. Four groups of rats were used: saline-injected controls, mild shock rats, severe shock rats, and rats pretreated with the specific neuronal reuptake blocker, desmethylimipramine (20 mg/kg) sixty minutes prior to the experiment. All rats were injected intravenously in the dorsal vein of the penis with small amounts of high specific

Figure 8.

FOCAL program used to perform standard channels ratio quench correction and calculate ^{3}H, ^{3}H-norepinephrine, and net norepinephrine uptake.

```
C-PS/8 FOCAL, 1971

01.10 T !!!"SINDATA",!;S S1=0;S S2=0;S S3=0;S S4=0;S S5=0;
01.30 A "HOW MANY DAYS SINCE DEC. 31, 1979?  "T;T !!
01.32 S ACT=500000/[FEXP((.301*(T+2832)/4475.63)*FLOG(10))]
01.33 T %6.02,"NET ACTIVITY OF STANDARD IS:"ACT,!!
01.35 A "ML ON COLUMN?  "ON;A "    ML OFF COLUMN?  "OFF;A "   GRINDING VOL?  "Q
01.36 T !!;A "BACKGROUND CPM?  "BCK;T !!
01.38 T "ENTER DATA FROM QUENCH SET STANDARDS",!
01.40 F I=1,7;D 2
01.50 G 4.10

02.10 A "CHANNEL B CPM?  "CHB;T !;A "STANDARD CHANNEL RATIO?  "SCR;T !
02.20 S EFF=100*(CHB-BCK)/(ACT-BCK);S SCR=SCR*.01
02.32 T %6.02,"PER CENT EFFICIENCY IS:",EFF,!!!
02.50 S S1=S1+SCR;S S2=S2+SCR^2
02.60 S S3=S3+EFF;S S4=S4+SCR*EFF;S S5=S5+EFF^2

04.10 S A1=(S3*S2-S4*S1)/(7*S2-S1^2)
04.20 S A2=(7*S4-S1*S3)/(7*S2-S1^2);S A=A2^2;S B=A1*A2
04.30 S D=7*S2-S1^2
04.40 S S=A2^2*S2+2*A2*A1*S1-2*A2*S4
04.50 S S=(S-2*A1*S3+S5)/7+A1^2
04.55 S EM=FSQT(7*S/D);S EB=FSQT(S*S2/D)
04.60 S R=(7*S4-S1*S3)/FSQT((7*S2-S1^2)*(7*S5-S3^2))
04.70 T !!,%6.02,"THE SLOPE IS"A2,"+/-"EM,!"THE INTERCEPT IS"A1,"+/-"EB
04.80 T !%6.02,"THE CORRELATION COEFFICIENT IS"R

05.10 A !!"HOW MANY EXPERIMENTAL SAMPLES?  "C
05.15 A !"SPECIFIC ACTIVITY (CI/MOLE NE) OF INCUBATION MEDIA?  "INC;T !
05.20 T !!"ENTER DATA FROM EXPERIMENTAL SAMPLES"!
05.30 F I=1,C;D 6
05.35 A !"ANY MORE?"QU;T !
05.36 I (QU-0Y)9.10,5.10

06.15 A "SAMPLE NAME:  "JUNK;T !
06.20 A "      CHANNEL B CPM?  "BDAT;T !
06.30 A "      STANDARD CHANNELS RATIO?  "SDAT;T !
06.36 A "      TISSUE WT. IN GRAMS?  ",X;T !
06.40 S P=A2*SDAT*.01+A1
06.42 S BDAT=BDAT-BCK
06.45 S BM=BDAT/(P/100)
06.50 S BNET=[BDAT/(P/100)]/(X*10)
06.54 S CAR=INC*2.2
06.55 S NOR=BNET*OFF*(Q+X)/(.4*ON*CAR)
06.60 T !,%6.02,"      THE PER CENT EFFICIENCY IS"P,!
06.65 T %8.02,"      THE NET DPM FOR THIS SAMPLE IS",!55,BM,!
06.70 T %8.02,"      THE NET DPM/100 MG FOR THIS SAMPLE IS ",!55,BNET,!
06.80 T %8.02,"      THE NET UPTAKE OF NE (PICOMOLES/100 MG) IS",!55,NOR,!!!

09.10 T !"THIS IS THE END OF THE LIQUID SCINTILLATION DATA PROGRAM"
```

activity (11 Ci/mmol) L-(7,8-^{3}H)-norepinephrine (20 μCi/kg). Therefore, rats were killed by decapitation ten minutes after injection with ^{3}H-norepinephrine. Hearts and spleens were removed, blotted, and assayed for norepinephrine and ^{3}H-norepinephrine content as described above. Data were expressed as dpm/gm tissue wet weight.

Preliminary experiments demonstrated that maximum uptake of labeled norepinephrine occurred within ten minutes of its injection. Saline control and five hour endotoxic rats were injected with 20 μCi/kg of ^{3}H-norepinephrine. Animals were selected at random either 5 or 10 minutes later and decapitated. Hearts and spleens were assayed for ^{3}H-norepinephrine. In the saline series, there was no difference in ^{3}H-norepinephrine content of hearts between the 5 minute (12675 ± 816 dpm/gm; n=5) and 10 minute (11518 ± 1142 dpm/gm; n=5) circulation time groups. In saline control spleens, the 5 minute group (2168 ± 151 dpm/gm; n=4) was no different from the 10 minute group (2315 ± 309 dpm/gm; n=5). Similar results were found in tissues from endotoxic rats. Cardiac ^{3}H-norepinephrine in endotoxic rats was 13871 ± 929 dpm/gm (n=10) for 5 minutes of circulation time and 14077 ± 1254 dpm/gm (n=10) in the 10 minute group. Spleen ^{3}H-norepinephrine in the endotoxic group was 1642 ± 270 dpm/gm (n=10) in the 5 minute group and 1382 ± 359 dpm/gm (n=8) in the 10 minute group. Statistical analysis was performed between each set of 5 and 10 minute groups (e.g. spleens of saline treated rats sacrificed 5 versus 10 minutes after ^{3}H-norepinephrine) by an independent Student's t test. No significant differences in ^{3}H-norepinephrine content existed between the 5 and 10

minute circulation time groups for either control or endotoxic rats. Thus, for *in vivo* uptake experiments, tissue sampling at 10 minutes after ^{3}H-norepinephrine injection was considered to measure total uptake of the labeled amine in both control and endotoxic series of rats.

2. *IN VITRO* REUPTAKE MEASUREMENT

Norepinephrine reuptake was assessed by measuring the amount of ^{3}H-norepinephrine incorporated in tissue slices incubated *in vitro* in two groups of rats: saline-injected controls and severely shocked rats. After decapitation, hearts and spleens were removed from both groups of rats. Tissues were sliced at 4° C with a Stadie-Riggs tissue slicer to a thickness of 0.5 mm. Approximately 100 mg samples were placed in flasks containing 4.9 ml of Krebs Ringer Bicarbonate buffer. The samples were preincubated for 10 minutes at 37° C in a Dubnoff metabolic shaker while being gassed with 95% oxygen and 5% carbon dioxide. Norepinephrine and ^{3}H-norepinephrine were added in a total volume of 0.1 ml to bring the final concentration of norepinephrine to 10^{-7} M and 0.1 μCi/ml. Tissues were then incubated for an additional 0, 10, 20, or 30 minutes. At the end of the incubation period tissues were removed from the incubation media, rinsed twice in cold (4° C) Krebs Ringer Bicarbonate buffer, frozen rapidly between tongs precooled in liquid nitrogen, and subsequently stored in liquid nitrogen. Samples were analyzed for total ^{3}H-norepinephrine content as described above. Total norepinephrine retention (the amount of norepinephrine that was taken up and retained as unmetabolized norepinephrine) was expressed in pmol/100

mg tissue wet weight. The value was calculated as the quotient of the tissue ^{3}H-norepinephrine content (dpm/mg tissue) and the specific activity of the incubation media (dpm/pmol norepinephrine). In some experiments an additional sample of tissues was incubated for 30 minutes at 37° C in the presence of 10^{-3} M desmethylimipramine, a potent Uptake 1 inhibitor, to characterize the measured reuptake as neuronal in nature. A number of dose response curves were generated that demonstrated over 90% of the ^{3}H retention was eliminated in both control and endotoxic rats by incubation of the tissues with 10^{-3} M desmethylimipramine.

G. Norepinephrine Turnover Measurement

Norepinephrine turnovers were measured in hearts and spleens by the ^{3}H-norepinephrine decay method (26). This technique relies on the neuronal uptake of norepinephrine and its rapid equilibration with endogenous stores. Once the tissue pool of norepinephrine has been labeled, ^{3}H-norepinephrine is released along with the unlabeled norepinephrine. Replenishment of the pool by biosynthesis of unlabeled norepinephrine decreases the specific activity of norepinephrine in the tissue. Thus, the greater the nerve activity, the more rapidly ^{3}H-norepinephrine disappears from the tissue. If the total pool of norepinephrine remains constant, the reduction of norepinephrine specific activity obeys first order kinetics and rate constants of decay are calculated. During endotoxin shock the depletion of norepinephrine from peripheral organs is dose and time dependent (120). Therefore, in

the present experiments low dose endotoxin treatment (6.7 mg/kg or approximately 2 mg/300 gm rat; preliminary testing indicated a lethality of 5% at 24 hours) was used to minimize the tissue depletion of norepinephrine.

For turnover studies, rats were housed individually as described above for three days prior to and at all times during the 12 hour experiment. Two experimental groups were used: 1) endotoxic rats (6.7 mg/kg) and 2) saline-injected controls. Rats (321 $\pm$ 3 gm) were injected intravenously with high specific activity (11 Ci/mmol) l-(7,8-^{3}H)-norepinephrine (20 µCi/kg) one minute prior to injection with either saline or endotoxin. The rats were killed at preselected times by decapitation. Each time point of the turnover study represents four to seven animals from each experimental group. Hearts and spleens were rapidly removed, blotted, and frozen between brass tongs precooled in liquid nitrogen. The samples were subsequently stored in liquid nitrogen until analyzed for norepinephrine content and the specific activity of norepinephrine as described above. The specific activity of norepinephrine (dpm/µg norepinephrine) was calculated as the quotient of ^{3}H-norepinephrine present in the sample (dpm/gm tissue) and tissue norepinephrine content (µg norepinephrine/gm tissue).

The specific activity of tissue norepinephrine was plotted versus time on a semi-logarithmic scale after injection of a tracer dose of ^{3}H-norepinephrine. The decay of specific activity versus time obeys first-order kinetics (and thus describes a straight line on a

semi-logarithmic plot) when low doses of high specific activity ^{3}H-norepinephrine are injected (25). This was the case in the present experiments. A regression line was calculated by the method of least squares. Kinetic data were determined as follows (12,27,105):

where: NE = endogenous concentration of norepinephrine in the tissue (µg/gm tissue wet weight);

NE^* = concentration of ^{3}H-norepinephrine divided by NE at any time, t (i.e. the specific activity of norepinephrine in the tissue in dpm/µg norepinephrine);

NE^*_o = NE^* at time zero (i.e. the time of tracer injection);

k = the rate constant of NE^* decay in the tissue, i.e. the fraction of the norepinephrine pool replaced per unit time (hr^{-1});

T_t = turnover time, i.e. the time required to synthesize the amount of norepinephrine stored in the tissue (hr); T_t is the reciprocal of the rate constant, k;

T_r = turnover rate, i.e. the rate at which the norepinephrine pool is renewed per unit time (µg norepinephrine/gm tissue wet weight)/hr);

$T_{1/2}$ = time required for NE^*_o to decline by one half;

Then, according to first order kinetics:

$$dNE^*/dt = -k(NE^*);$$

integration yields:

$$NE^* = (NE^*_o)e^{-(k)(t)}$$

and,

$$\log NE^* = \log NE^*_o - 0.434(k)(t).$$

The equation is now of the form: $y=mx+b$, so

$0.434(k)$ = the slope,

and

$\log NE^*_o$ = the y intercept

of the graph $\log NE^*$ versus time.

Then by substituting $T_{1/2}$ for t and $(1/2)NE^*$ for NE^*_o in the integrated form of the equation above,

$$T_{1/2} = (0.693)/k,$$

$$T_t = 1/k = (1.44)T_{1/2},$$

and

$$T_r = (NE)k.$$

Although the graphs depict the specific activity plotted versus time on semi-logarithmic coordinates, the values of the slope and rate constant (k) were calculated on the basis of the logarithm of the specific activity to conform to the usual convention.

H. Statistical Analysis

Unless otherwise stated, all data were expressed as mean $\pm$ SEM. Statistical significance was tested by two methods except where noted. For comparison of two groups an independent Student's t test was used. The comparison of more than two groups was accomplished with an independent analysis of variance. Significant F tests were followed by the mean separation technique of a least significant difference (LSD).

A p value of less than 0.05 was accepted as achieving a significant difference.

For norepinephrine turnover experiments, differences between the slopes of the regression lines were tested with a Student's t test, using the standard error of sample regression. Differences in norepinephrine content were tested by an independent analysis of variance.

CHAPTER V

RESULTS

A. Demonstration of Norepinephrine Depletion during Endotoxin Shock

Rats were injected with saline or endotoxin (5 mg/300 gm rat) and sacrificed at the earlier of two times: five hours after injection or near death. Plasma glucose was measured from a mixed arterial/venous blood sample; hearts and spleens were analyzed for norepinephrine and epinephrine content. Table III depicts the results of these experiments for all endotoxic rats versus saline controls. The plasma glucose concentration was significantly reduced in both groups of endotoxic rats ($p<0.01$). Cardiac norepinephrine was significantly reduced ($p<0.01$) in the endotoxic group from a control value of 0.985 ± 0.05 µg/gm (mean ± SEM) to 0.600 ± 0.06 µg/gm. Similarly, splenic norepinephrine content was depressed in the endotoxic group: 1.214 ± 0.07 µg/gm for controls and 0.553 ± 0.07 µg/gm for endotoxic rats ($p<0.01$).

Epinephrine content is reported only for cardiac samples. The trihydroxyindole technique limits the quantitation of epinephrine in a mixed norepinephrine/epinephrine sample to those epinephrine values that are greater than 5% of the norepinephrine content for that sample (29). Thus, in samples that contain relatively high amounts of norepinephrine (as in spleens from control rats) the epinephrine values are usually too

TABLE III. PLASMA GLUCOSE AND TISSUE CATECHOLAMINE CONTENT OF ENDOTOXIC AND CONTROL RATS

	Saline Control	Endotoxic
CARDIAC NOREPINEPHRINE (µg/gm)	0.985 ± 0.05	0.600 ± 0.06 *
Plasma Glucose (mg/dl)	90 ± 3	53 ± 7 *
N	15	21
CARDIAC EPINEPHRINE (µg/gm)	0.087 ± 0.02	0.187 ± 0.03 *
Plasma Glucose (mg/dl)	92 ± 5	53 ± 7 *
N	5	21
SPLENIC NOREPINEPHRINE (µg/gm)	1.214 ± 0.07	0.553 ± 0.07 *
Plasma Glucose (mg/dl)	90 ± 3	47 ± 7 *
N	11	14

Mean ± SEM;
* $p<0.01$ endotoxic group compared to saline control by independent Student's t test;

low to allow quantitative significance. In the myocardium, however, the mean epinephrine content rose from a control value of 0.087 ± 0.02 µg/gm to 0.187 ± 0.03 µg/gm ($p<0.01$). Note that due to the limitations of the epinephrine measurement, only 5 of 15 control rats were included in the calculation of epinephrine values.

To assess the severity of shock on the catecholamine content of hearts and spleens, endotoxic rats were divided into mild and severe shock on the basis of their plasma glucose levels. Mild shock is denoted by a plasma glucose greater than 40 mg/dl; severe shock is denoted by a plasma glucose less than 40 mg/dl. The results of this differentiation are shown in Figures 9 and 10 for cardiac and splenic norepinephrine, respectively. In hearts (Figure 9), norepinephrine levels were significantly reduced 17% in mild shock rats ($p<0.01$). Severe shock rats demonstrated a more profound depletion in norepinephrine content (control: 0.985 ± 0.05 µg/gm; severe shock: 0.312 ± 0.04 µg/gm; $p<0.01$). This constituted a 68% reduction compared to control. Similar results (Figure 10) occurred in spleens from a different group of rats. Rats in mild shock displayed a moderate depletion of 36% compared to control (control: 1.21 ± 0.06 µg/gm; mild shock: 0.77 ± 0.08 µg/gm; $p<0.01$). The depletion was much more pronounced in severely shocked rats than in the mild shock group; norepinephrine content was reduced 77%, from the control level of 1.21 ± 0.06 µg/gm to 0.28 ± 0.03 µg/gm ($p<0.01$). The relationship between plasma glucose and norepinephrine content for all endotoxic rats is illustrated in Figures 11 and 12 for hearts and spleens, respectively.

Figure 9.

Mean ± SEM of cardiac norepinephrine content and plasma glucose concentration for control and endotoxic rats. Endotoxic rats were divided into mild and severe groups on the basis of their plasma glucose concentration (see text for details). Numbers of animals are indicated in parentheses. * $p<0.01$, ** $p<0.05$ compared to control by independent analysis of variance.

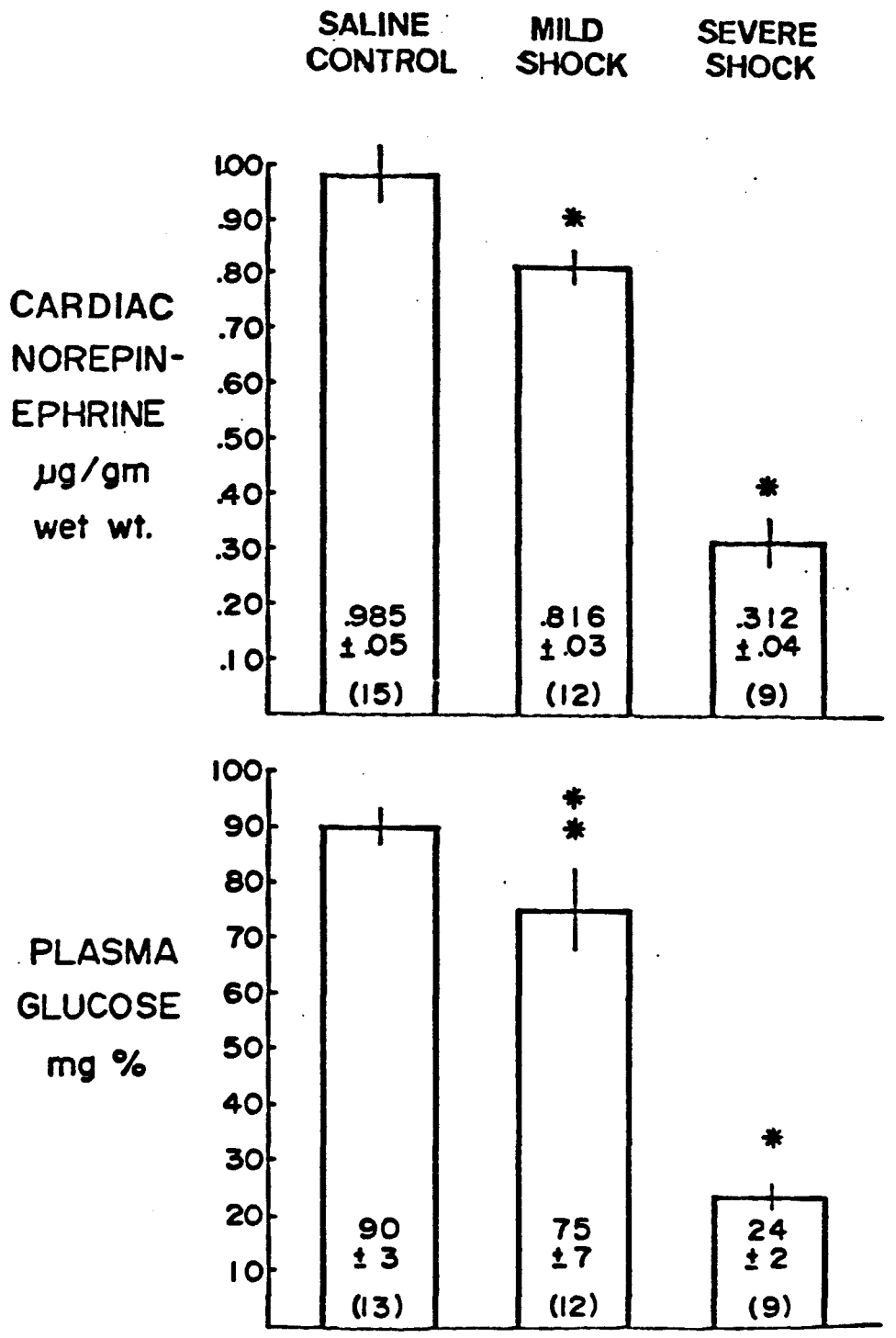
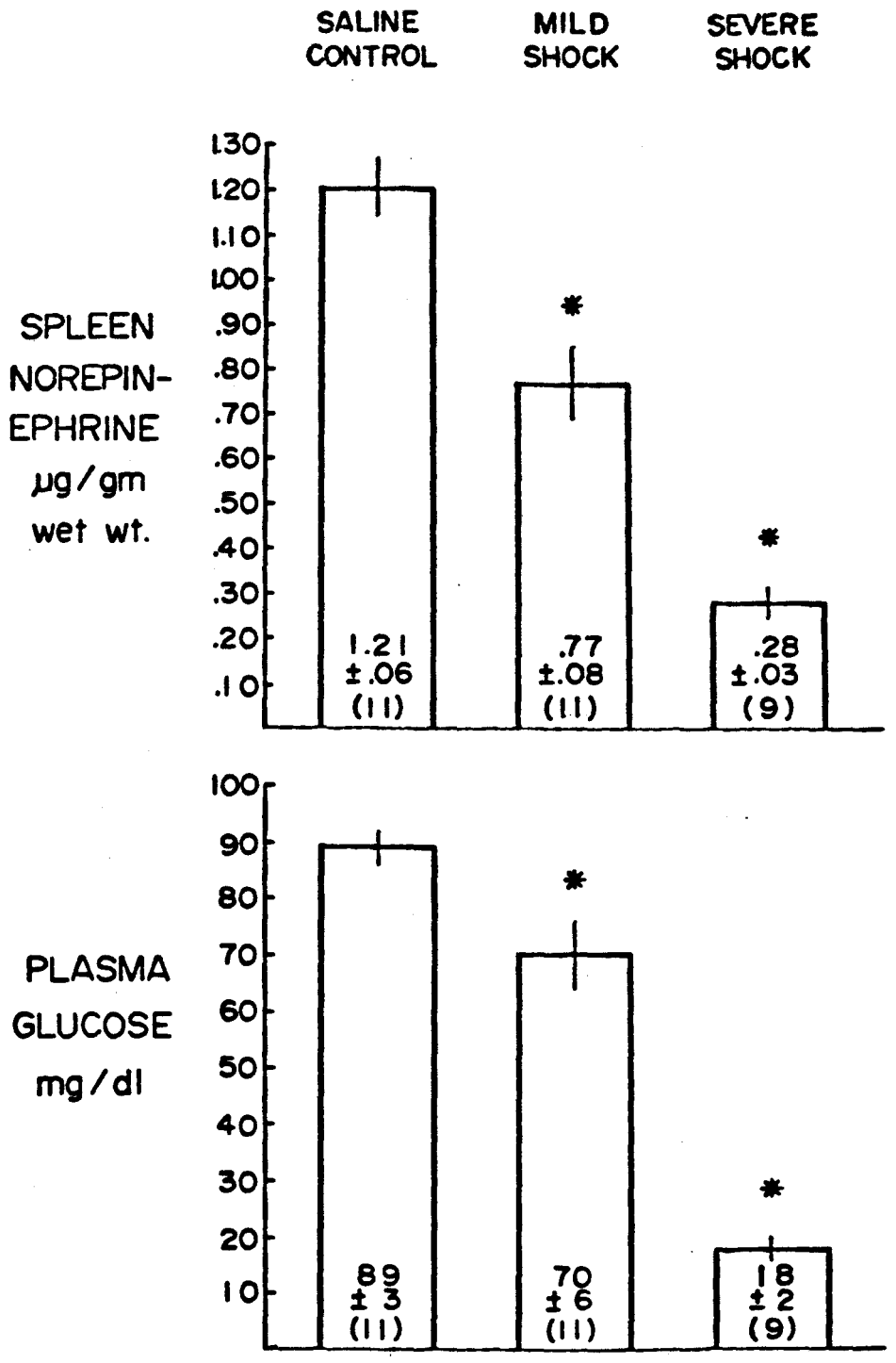

SALINE CONTROL
MILD SHOCK
SEVERE SHOCK
CARDIAC NOREPIN-EPHRINE µg/gm wet wt.
100
.90
.80
.70
.60
.50
.40
.30
.20
.10
.985 ± .05 (15)
.816 ± .03 (12)
.312 ± .04 (9)
PLASMA GLUCOSE mg %
100
90
80
70
60
50
40
30
20
10
90 ± 3 (13)
75 ± 7 (12)
24 ± 2 (9)

Figure 10.

Mean ± SEM of splenic norepinephrine content and plasma glucose concentration for control and endotoxic rats. Endotoxic rats were divided into mild and severe groups on the basis of their plasma glucose concentration (see text for details). Numbers of animals are indicated in parentheses. * $p<0.01$ compared to control by independent analysis of variance.


SALINE CONTROL
MILD SHOCK
SEVERE SHOCK
SPLEEN NOREPIN-EPHRINE µg/gm wet wt.
1.30
1.20
1.10
1.00
.90
.80
.70
.60
.50
.40
.30
.20
.10
1.21 ±.06 (11)
.77 ±.08 (11)
.28 ±.03 (9)
*
*
PLASMA GLUCOSE mg/dl
100
90
80
70
60
50
40
30
20
10
89 ±3 (11)
70 ±6 (11)
18 ±2 (9)
*
*

Linear regression analysis was performed to determine any correlation between the two parameters. For hearts (Figure 11), the equation of the regression line (of the form $y = mx + b$) was $y = 0.0063x + 0.267$. The correlation coefficient, r, was 0.73, and indicated a significant correlation ($p<0.01$) between plasma glucose and cardiac norepinephrine. Similar results were obtained in the spleen (Figure 12). The equation of the regression line was $y = 0.0082x + 0.167$, and demonstrated a significant correlation ($r=0.78$, $p<0.01$) between plasma glucose and splenic norepinephrine content during endotoxin shock.

B. Characterization of the Norepinephrine Depletion Phenomenon

1. THE SINGULAR ROLE OF HYPOGLYCEMIA ON NOREPINEPHRINE CONTENT IN HEART AND SPLEEN

To assess the role of low plasma glucose on the norepinephrine depletion of endotoxicosis, one group of rats was treated with insulin to lower plasma glucose concentration to varying degrees. Under light ether anesthesia rats were injected intravenously with various amounts (0 to 3.5 U/kg) of regular insulin (Eli Lilly and Co.) and sacrificed 90 minutes later. Plasma glucose was measured, and hearts and spleens were analyzed for catecholamine content. Figure 13 is a graph of injected insulin dose (U/kg) versus plasma glucose concentration (mg/dl) 90 minutes after insulin injection. The figure illustrates the range and distribution of insulin doses as well as the classic relationship between insulin and plasma glucose levels.

Figure 11.

Cardiac norepinephrine content is plotted against plasma glucose concentration for all endotoxic rats of Figure 9. Linear regression analysis indicates a significant correlation between cardiac norepinephrine and plasma glucose concentration ($r=0.73$, $p<0.01$). Equation (of the form $y = mx + b$) for the line is $y = 0.0063x + 0.267$.

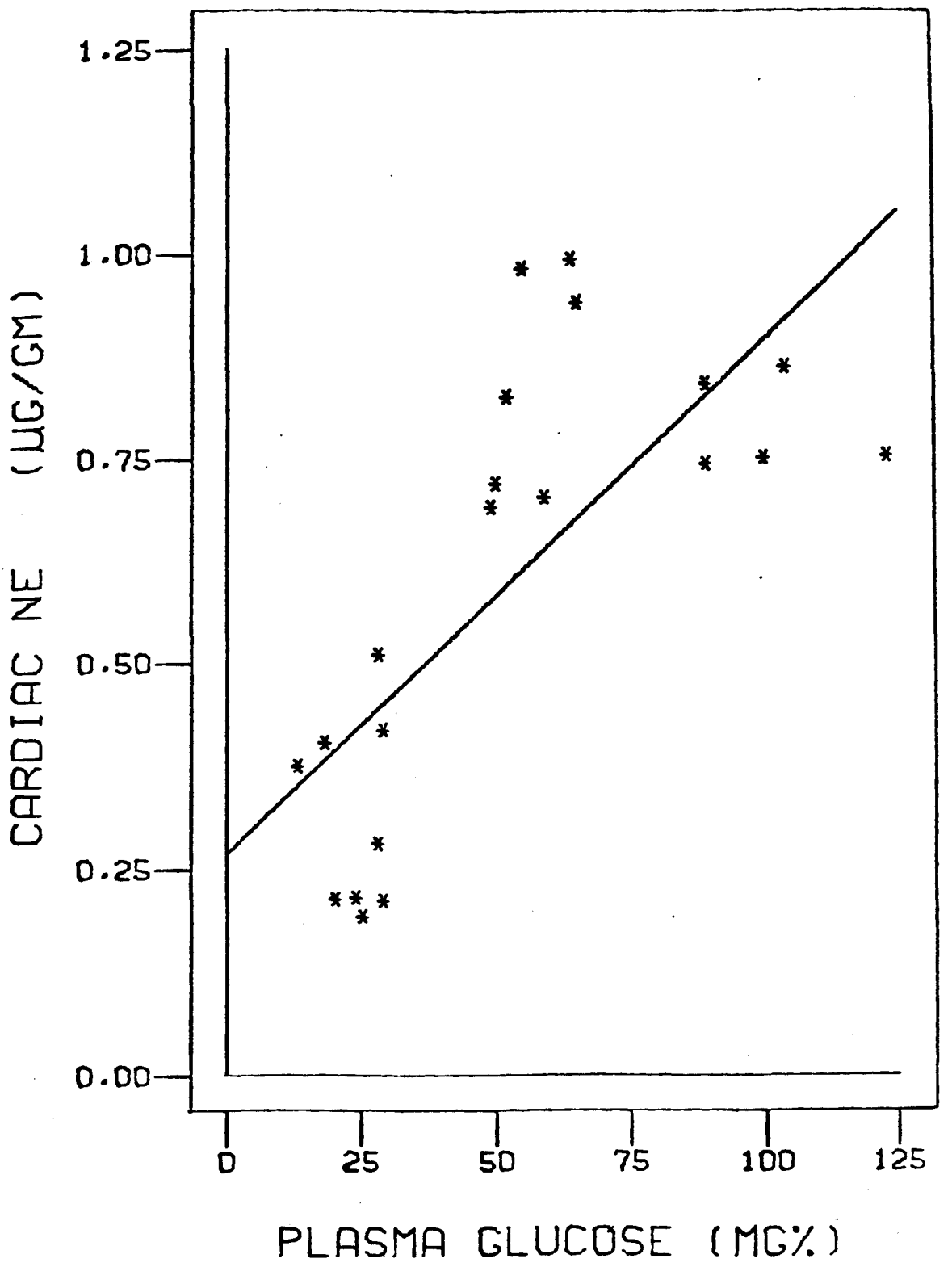

1.25
1.00
0.75
0.50
0.25
0.00
CARDIAC NE (UG/GM)
0
25
50
75
100
125
PLASMA GLUCOSE (MG%)

Figure 12.

Splenic norepinephrine content is plotted against plasma glucose concentration for all endotoxic rats of Figure 10. Linear regression analysis indicates a significant correlation between splenic norepinephrine and plasma glucose concentration ($r=0.78$, $p<0.01$). Equation (of the form $y = mx + b$) for the line is $y = 0.0082x + 0.167$.

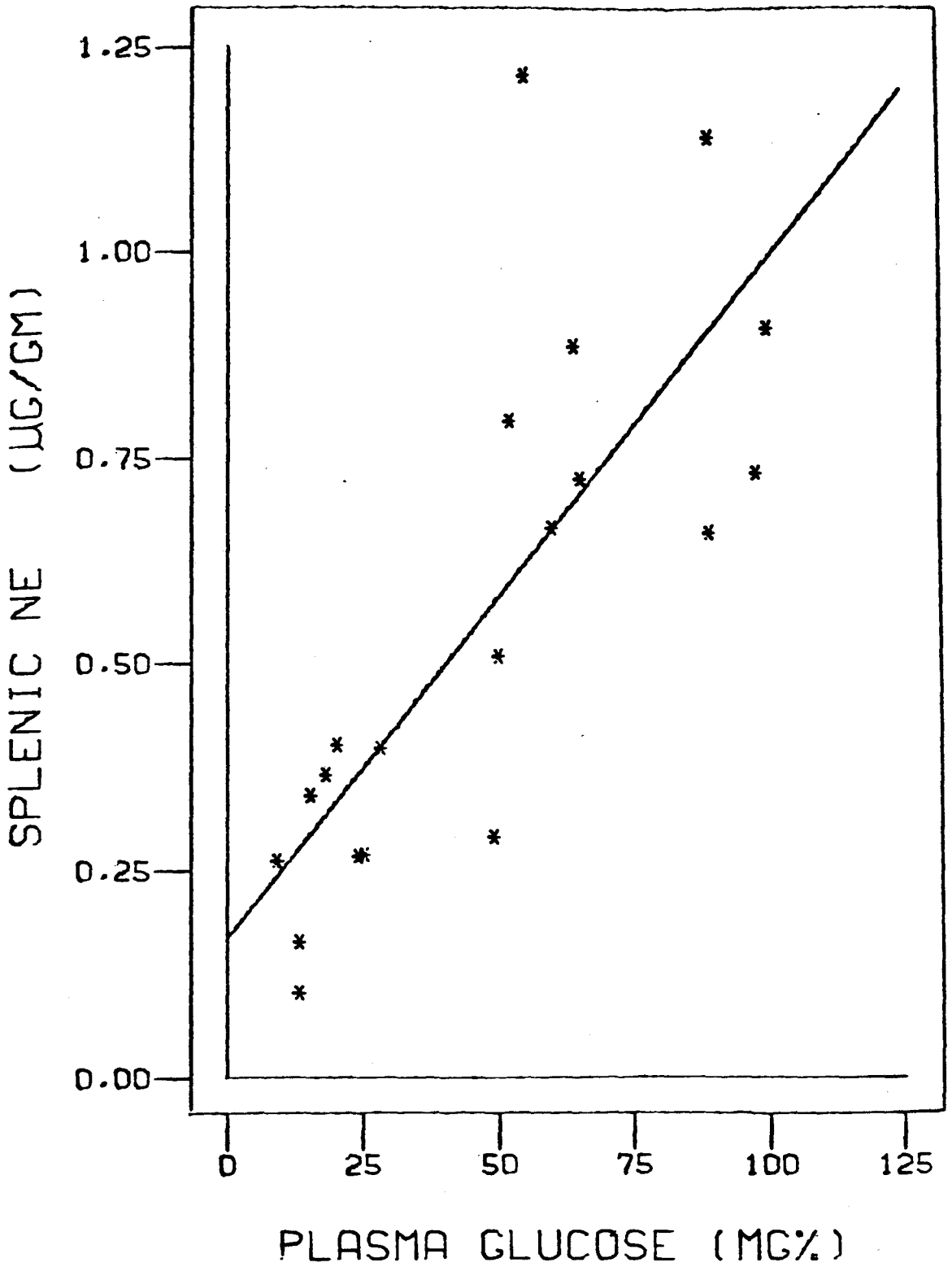
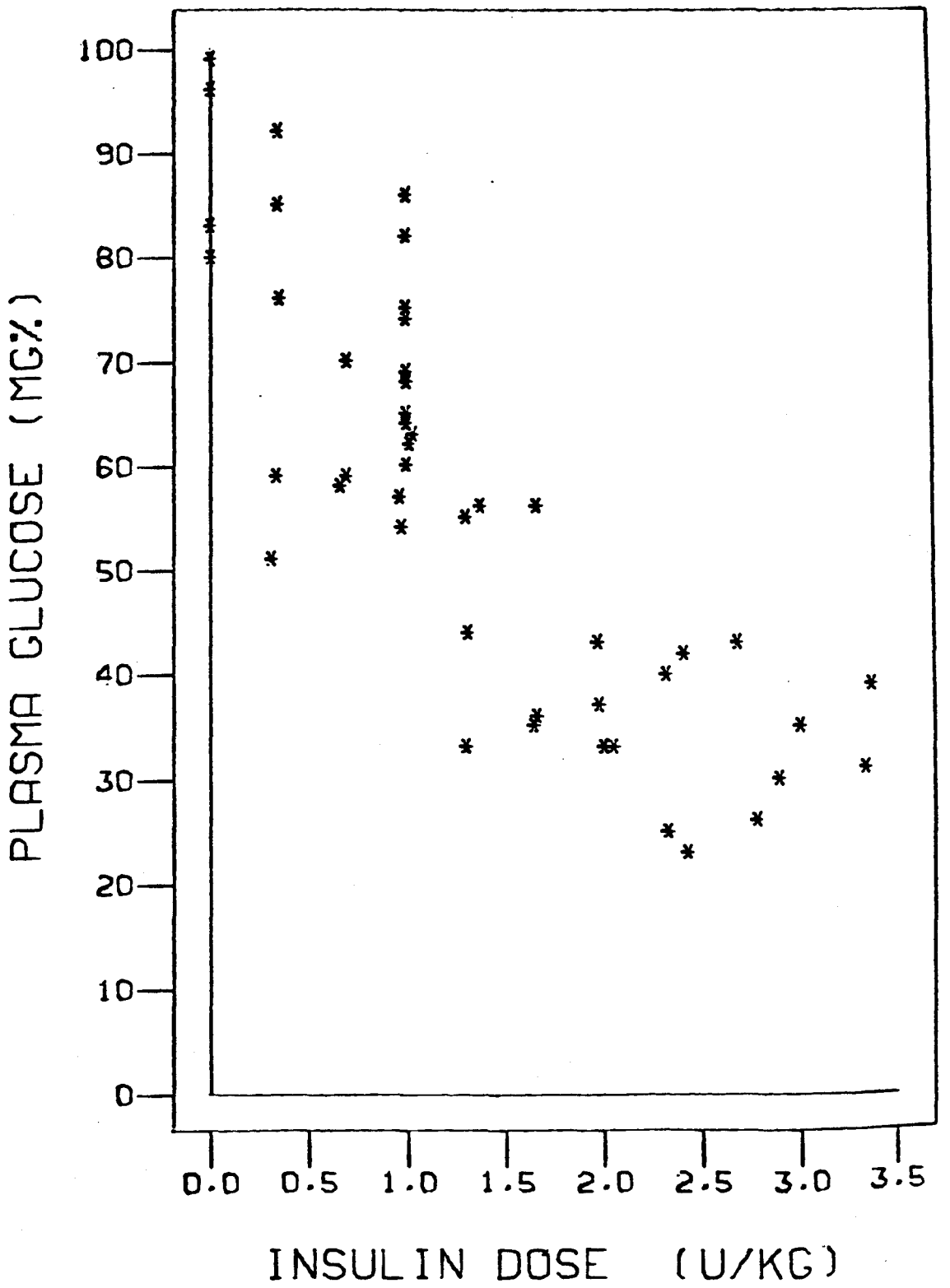

1.25
1.00
0.75
0.50
0.25
0.00
SPLENIC NE (UG/GM)
0
25
50
75
100
125
PLASMA GLUCOSE (MG%)

Figure 13.

Graph depicts the relationship between the injected dose of insulin (U/kg) and the plasma glucose concentration (mg/dl) 90 minutes after intravenous insulin injection.


PLASMA GLUCOSE (MG%)
100
90
80
70
60
50
40
30
20
10
0
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
INSULIN DOSE (U/KG)

The results of the experiments are shown in Table IV. For each organ, the data are first shown for the entire shock or insulin hypoglycemia group. The groups are then shown separated into mild and severe groups (insulin-treated rats were separated into mild and severe groups by the same criteria as endotoxic rats). The plasma glucose concentrations were comparable between shock and insulin groups. However during the mild phase, insulin hypoglycemia had a tendency toward lower plasma glucose than respective endotoxic samples (not statistically significant), while during the severe phase, the shock group demonstrated lower plasma glucose levels ($p<0.01$). Comparison between norepinephrine content for all animals in the shock and insulin groups demonstrated that for both hearts and spleens the norepinephrine content of the insulin hypoglycemic group always contained greater amounts of norepinephrine than the shock group ($p<0.01$ in both cases). Similar results were found when the total groups were separated into mild and severe groups. Cardiac norepinephrine was significantly reduced 12% in the mild shock group ($p<0.05$) and 64% in the severe shock group ($p<0.01$) compared to the respective insulin hypoglycemic groups. Similarly, splenic norepinephrine was significantly reduced 38% in the mild shock group and 75% in the severe shock group compared to the respective insulin hypoglycemic groups ($p<0.01$ in both cases).

Closer inspection of organ norepinephrine content in the insulin hypoglycemic group revealed that no significant reduction from saline controls occurred. In the spleen, norepinephrine content in saline

TABLE IV. COMPARISON BETWEEN ENDOTOXIN- AND INSULIN-INDUCED HYPOGLYCEMIA ON HEART AND SPLEEN NOREPINEPHRINE CONTENT

	N	Tissue Norepinephrine (µg/gm)	Plasma Glucose (mg/dl)
HEART			
All Shock	21	0.600 ± 0.06	53 ± 7
		*	
All Insulin Hypoglycemia	41	0.905 ± 0.03	52 ± 4
Mild Shock	12	0.816 ± 0.03	75 ± 7
		**	
Mild Insulin Hypoglycemia	27	0.924 ± 0.03	63 ± 3
Severe Shock	9	0.312 ± 0.04	24 ± 2
		*	*
Severe Insulin Hypoglycemia	14	0.868 ± 0.05	33 ± 1
SPLEEN			
All Shock	20	0.553 ± 0.07	47 ± 7
		*	
All Insulin Hypoglycemia	33	1.225 ± 0.07	59 ± 4
Mild Shock	11	0.772 ± 0.08	70 ± 6
		*	
Mild Insulin Hypoglycemia	24	1.254 ± 0.09	69 ± 3
Severe Shock	9	0.284 ± 0.04	18 ± 2
		*	*
Severe Insulin Hypoglycemia	9	1.149 ± 0.14	31 ± 2

* $p<0.01$, ** $p<0.05$ for shock versus insulin hypoglycemia group by independent Student's t test;

controls was 1.214 ± 0.07 µg/gm. This was not significantly different from either the mild insulin hypoglycemic group (1.254 ± 0.09 µg/gm) or the severe insulin hypoglycemic group (1.149 ± 0.14 µg/gm). In the heart, norepinephrine content of saline controls was 0.985 ± 0.05 µg/gm. There was no statistical reduction in either the mild (0.924 ± 0.03 µg/gm) or severe insulin hypoglycemic group (0.868 ± 0.05 µg/gm). Figures 14 and 15 illustrate the relationship between plasma glucose and norepinephrine content for hearts and spleens of all insulin hypoglycemic rats. Linear regression analysis was performed by the method of least squares. The equation of the regression line (of the form $y = mx + b$) for cardiac norepinephrine versus plasma glucose (Figure 14) was $y = 0.0014x + 0.833$. The correlation coefficient, r (0.15), was not significantly different from 0 ($p>0.05$); no correlation between cardiac norepinephrine content and plasma glucose occurred. Similar results were obtained in the spleen (Figure 15). The equation of the regression line was $y = -0.0002x + 1.238$. No correlation between splenic norepinephrine and plasma glucose was evident ($r=-0.01$, $p>0.05$).

Cardiac epinephrine was also quantitated in the insulin hypoglycemic rats. Epinephrine content of saline-injected controls was 0.087 ± 0.02 µg/gm (n=5). Mild insulin hypoglycemia significantly raised cardiac epinephrine levels 10% (0.096 ± 0.01 µg/gm; n=24); severe insulin hypoglycemia resulted in a marked 115% increase in cardiac epinephrine levels (0.189 ± 0.04 µg/gm). Figure 16 depicts the relationship between plasma glucose and cardiac epinephrine for all insulin hypoglycemic rats. The equation of the regression line was y =

Figure 14.

Graph depicts cardiac norepinephrine content versus plasma glucose concentration for all rats treated with insulin. Linear regression analysis indicates no correlation between norepinephrine content and plasma glucose concentration ($r = 0.15$, $p > 0.05$). Equation of the line (of the form $y = mx + b$) is $y = 0.0014x + 0.833$.

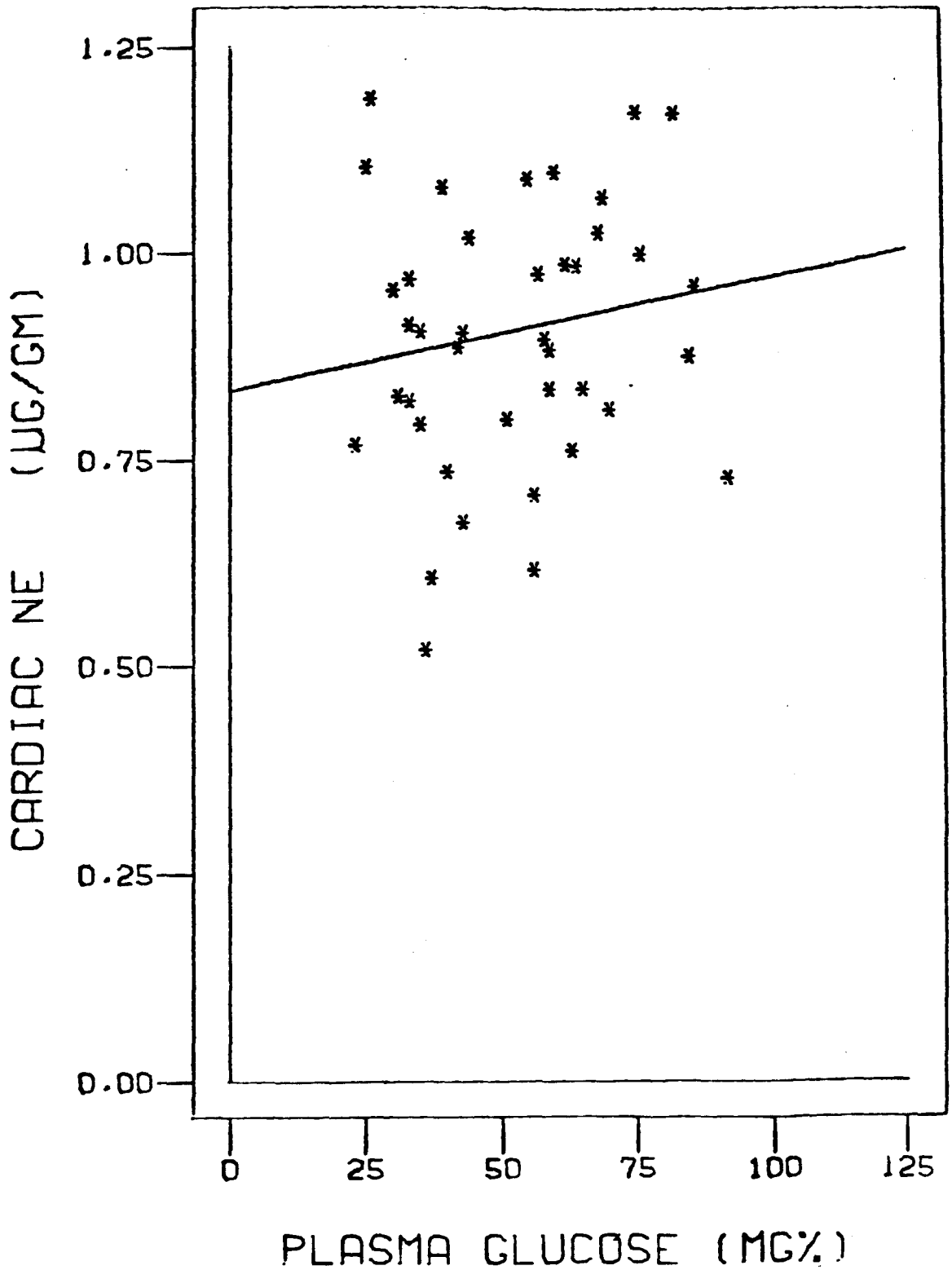

1.25
1.00
0.75
0.50
0.25
0.00
CARDIAC NE (UG/GM)
0
25
50
75
100
125
PLASMA GLUCOSE (MG%)

Figure 15.

Graph depicts splenic norepinephrine content versus plasma glucose concentration for all rats treated with insulin. Linear regression analysis indicates no correlation between norepinephrine content and plasma glucose concentration ($r = -0.01$, $p > 0.05$). Equation of the line (of the form $y = mx + b$) is $y = -0.0002x + 1.238$.

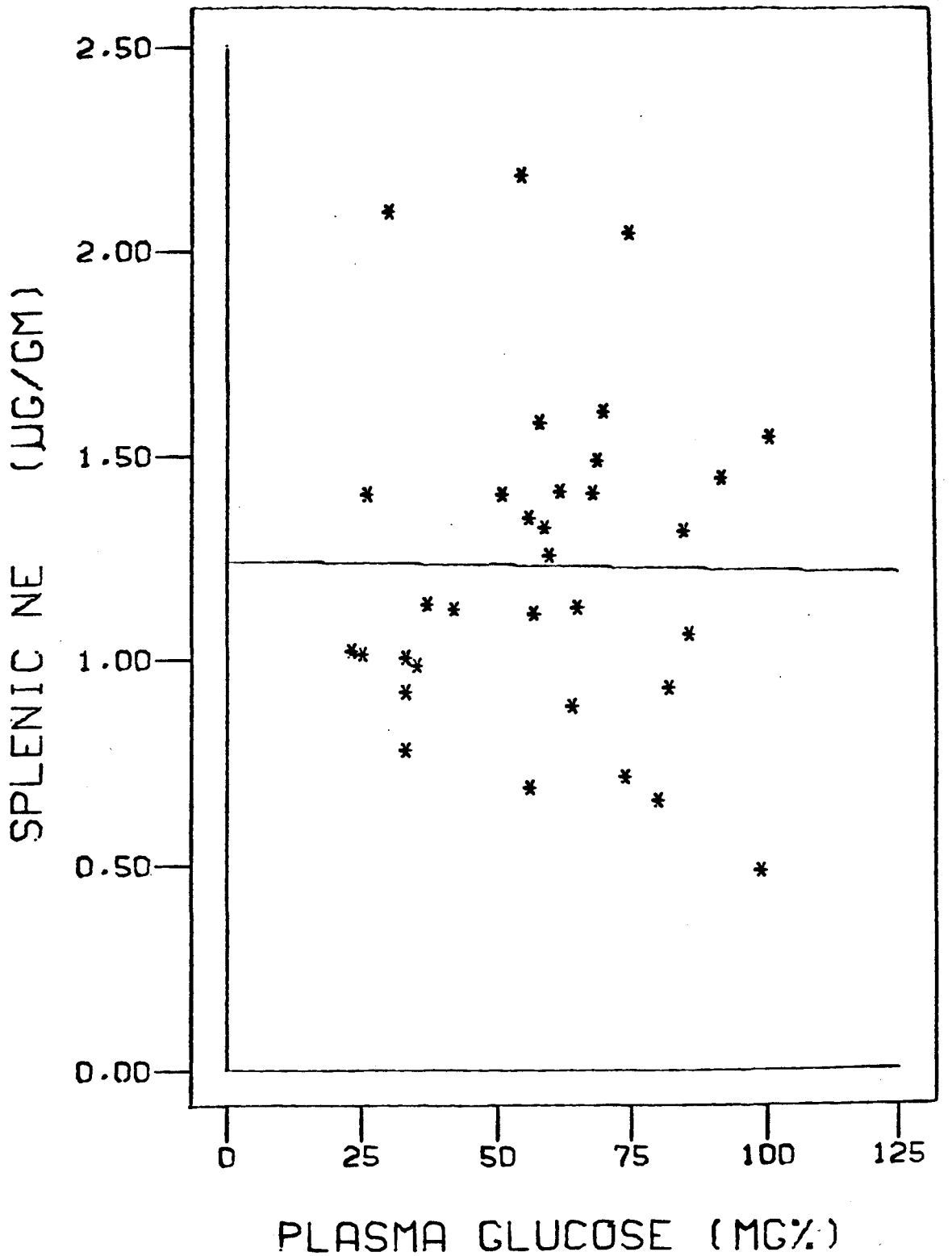
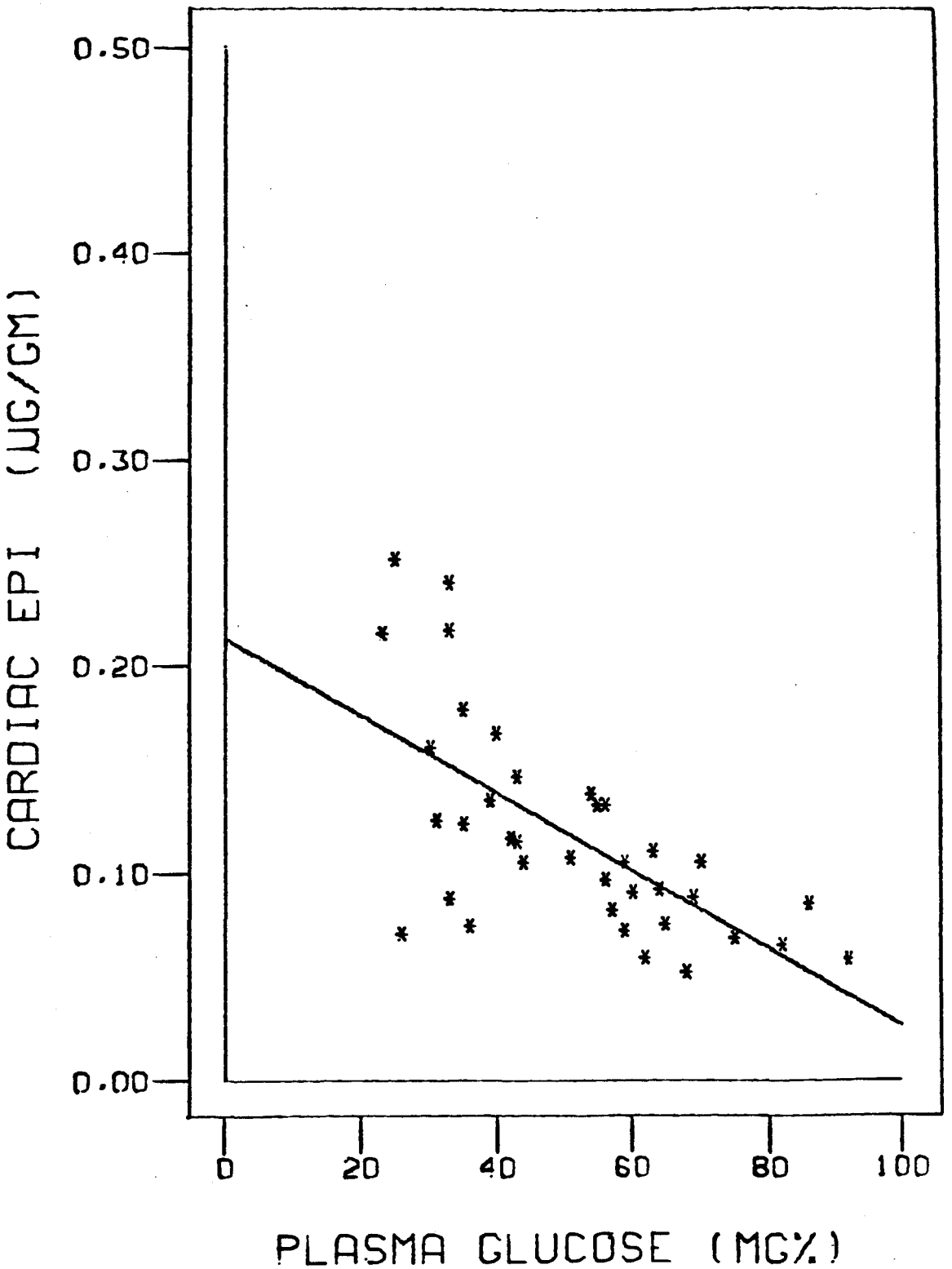

2.50
2.00
1.50
1.00
0.50
0.00
SPLENIC NE (UG/GM)
0
25
50
75
100
125
PLASMA GLUCOSE (MG%)

Figure 16.

Graph depicts cardiac epinephrine content versus plasma glucose concentration for all rats treated with insulin. Linear regression analysis indicates a significant negative correlation between norepinephrine content and plasma glucose concentration ($r = -0.66$, $p<0.01$). Equation of the line (of the form $y = mx + b$) is $y = -0.0019x + 0.213$.


0.50
0.40
0.30
0.20
0.10
0.00
CARDIAC EPI (UG/GM)
0
20
40
60
80
100
PLASMA GLUCOSE (MG%)

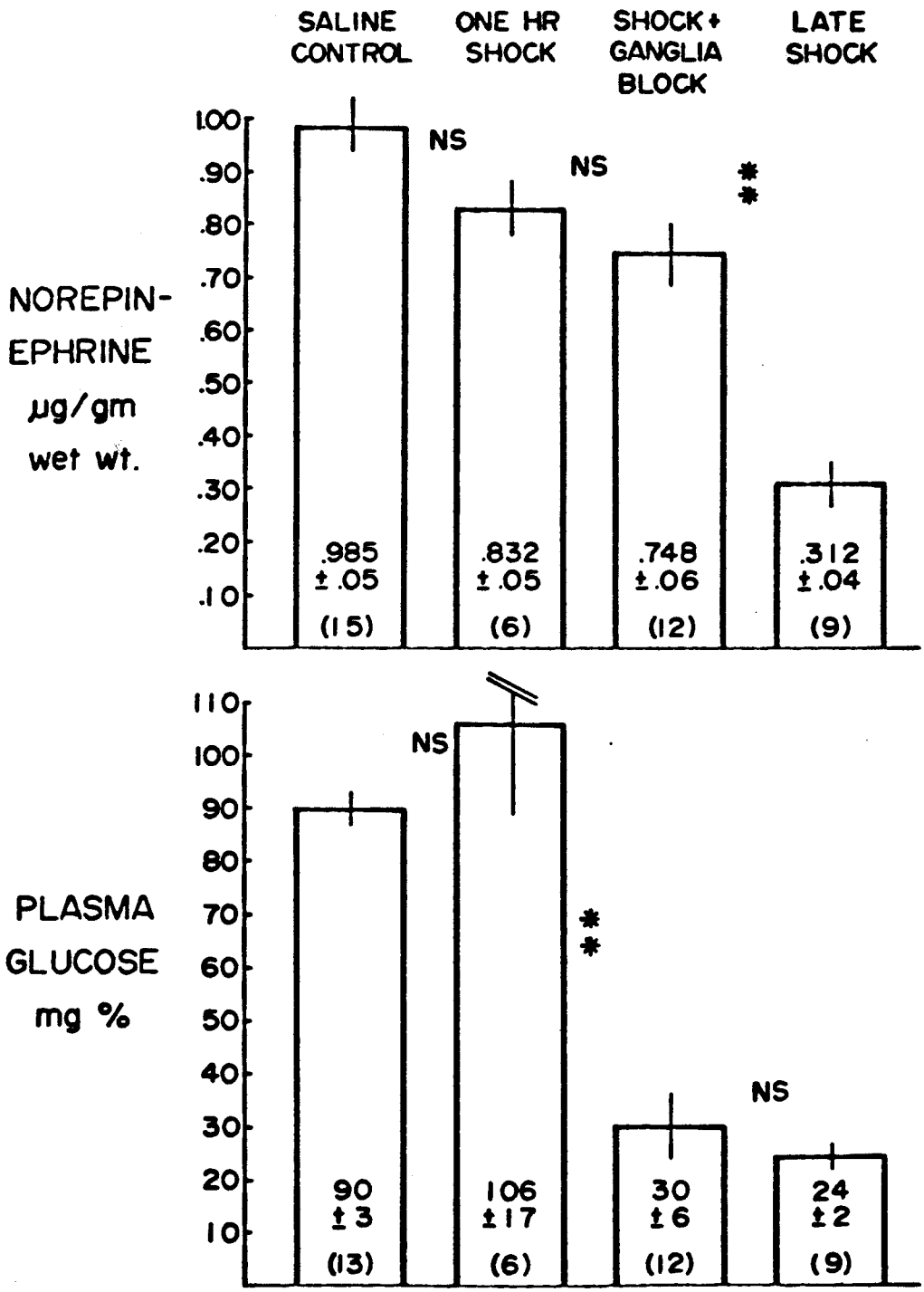
-0.0019x + 0.213. A significant negative correlation ($r=-0.656$; $p<0.01$) existed between cardiac epinephrine and plasma glucose for insulin hypoglycemic rats.

2. THE ROLE OF NEURAL MEDIATION IN THE NOREPINEPHRINE DEPLETION OF ENDOTOXIC SHOCK

To test the hypothesis that the norepinephrine depletion was dependent on neural activity, rats were injected intravenously with the ganglionic blocker, chlorisondamine (5 mg/kg), one hour after endotoxin injection (5 mg/300 gm rat). Preliminary experiments showed that pretreatment or cotreatment of chlorisondamine sensitized rats to endotoxin lethality (rats usually died while still under ether anesthesia or within five to ten minutes). Therefore, ganglionic blockade was administered one hour after endotoxin (post-hypotensive phase). This regimen was tolerated better by rats, and the time to death of the ganglionic blockade group (176 $\pm$ 21 min) was not significantly different than the endotoxic rats without ganglionic blockade (169 $\pm$ 35 min). Figure 17 illustrates the plasma glucose and cardiac norepinephrine for these experiments. An additional group of endotoxic rats was sacrificed at one hour after endotoxin to act as a control for rats with ganglionic blockade. Ganglionic blockade effectively eliminated any norepinephrine depletion after one hour post-endotoxin (0.832 $\pm$ 0.05 µg/gm for one hour of endotoxin; 0.748 $\pm$ 0.04 µg/gm for endotoxin plus ganglionic blockade). Plasma glucose

Figure 17.

Illustration of cardiac norepinephrine content and plasma glucose concentration in control, endotoxic, and endotoxic rats pretreated with chlorisondamine (5 mg/kg). Within the bars the mean ± SEM are shown. Number of rats is illustrated in parentheses. ** $p<0.01$ versus adjoining bar by independent analysis of variance.


SALINE CONTROL
ONE HR SHOCK
SHOCK + GANGLIA BLOCK
LATE SHOCK
NOREPIN-EPHRINE µg/gm wet wt.
1.00
.90
.80
.70
.60
.50
.40
.30
.20
.10
NS
NS
**
.985 ± .05 (15)
.832 ± .05 (6)
.748 ± .06 (12)
.312 ± .04 (9)
PLASMA GLUCOSE mg %
110
100
90
80
70
60
50
40
30
20
10
NS
**
NS
90 ± 3 (13)
106 ± 17 (6)
30 ± 6 (12)
24 ± 2 (9)

levels in the ganglionic blockade group (30 ± 6 mg/dl) reached levels as low as the severe shock group (24 ± 2 mg/dl).

3. EFFECT OF ADRENERGIC MANIPULATION ON ENDOTOXIN-INDUCED LETHALITY AND NOREPINEPHRINE DEPLETION

Adrenergic blockade and sympathectomy have been shown to have variable effects on the pathogenesis of endotoxin shock. To further investigate this area, rats were treated with either postsynaptic adrenergic blocking agents or chemically sympathectomized to prevent presynaptic release of norepinephrine. In the first set of experiments rats were treated with either an alpha adrenergic blocking agent (phentolamine) or beta adrenergic blocking agent (propranolol) and then subjected to the present shock model. In awake rats, phentolamine (3 mg/kg) or propranolol (4 mg/kg) was injected intraperitoneally 15 minutes before endotoxin was injected intravenously (5 mg/300 gm rat) under light ether anesthesia. A control group of rats was injected with only endotoxin. Rats were killed 5 hours later or near death. Results of these experiments are shown in Table V. All phentolamine plus endotoxin rats lived for the maximum five hours (300 ± 0 min) of the experiment. This was significantly longer than either the endotoxin alone group (277 ± 12 min) or the propranolol plus endotoxin group (18 ± 1 min). Propranolol significantly decreased time to death compared to the endotoxin alone group. Although plasma glucose was no different between endotoxin and propranolol plus endotoxin in this series, phentolamine plus endotoxin rats had significantly higher glucose levels

TABLE V. EFFECTS OF PROPRANOLOL OR PHENTOLAMINE PRETREATMENT ON ENDOTOXIN SHOCK IN THE RAT

	Endotoxin Alone	Propranolol Plus Endotoxin	Phentolamine Plus Endotoxin
Plasma Glucose (mg/dl)	51 ± 14 (8)	43 ± 5 (7)	*# 78 ± 5 (8)
Time to Death (minutes)	277 ± 12 (8)	* 18 ± 1 (12)	*# 300 ± 0 (11)
Cardiac Norepinephrine (μg/gm)	0.617 ± 0.10 (8)	0.761 ± 0.05 (7)	* 0.853 ± 0.07 (8)
Splenic Norepinephrine (μg/gm)	0.520 ± 0.11 (7)	0.812 ± 0.07 (7)	* 0.887 ± 0.13 (7)

Values are mean ± SEM. The number of samples is in parentheses below each value.
* $p<0.05$ versus endotoxin alone,
# $p<0.05$ versus propranolol plus endotoxin,
by independent analysis of variance.

than either of the other groups. Norepinephrine content in both the heart and spleen was statistically greater in the phentolamine plus endotoxin group than in the endotoxin alone group. However, norepinephrine content of hearts and spleens in propranolol-treated rats was intermediate and no different than in either of the other two groups.

The effects of blunted release of norepinephrine was next investigated by chemically sympathectomizing rats before endotoxin challenge. Animals sympathectomized with 6-hydroxydopamine contain minimal norepinephrine in peripheral sympathetic nerve endings, and higher than normal catecholamine levels in adrenal medullary tissue (norepinephrine and epinephrine). Because norepinephrine has relatively more alpha adrenergic effect and epinephrine has more beta adrenergic effect, chemical sympathectomy would accentuate beta adrenergic activity and minimize alpha adrenergic activity. In light of the phentolamine and propranolol experiments, 6-hydroxydopamine is hypothesized to decrease alpha adrenergic stimulation of sympathetically innervated tissues, leave adrenal medullary secretion of catecholamines intact, and significantly protect against endotoxic lethality. Under light ether anesthesia, rats were injected intravenously with phentolamine (10 mg/kg) and then either saline or 6-hydroxydopamine (80 mg/kg). 6-hydroxydopamine was weighed out as the free base of the amine complexed with hydrobromide and dissolved in saline containing 1% ascorbate. Phentolamine was administered to minimize the blood pressure increase that occurred after 6-hydroxydopamine injection and subsequent

norepinephrine liberation. Preliminary experiments indicated that this regimen of treatment with 6-hydroxydopamine reduced cardiac norepinephrine levels by 88% and splenic norepinephrine levels by 93%. Endotoxin was administered 48 hours later. The lethality data is illustrated in Table VI. Chemical sympathectomy significantly reduced the lethality associated with endotoxin injection. The protective effect of sympathectomy was first demonstrated at 6 hours after endotoxin and was present for the duration of the study up to 24 hours post endotoxin.

C. Norepinephrine Reuptake

1. IN VIVO ^{3}H-NOREPINEPHRINE UPTAKE IN ENDOTOXIC SHOCK

The results of *in vivo* assessment of the reuptake process in endotoxicosis are presented in Tables VII and VIII for hearts and spleens, respectively. Four groups of rats were evaluated: 1) saline-injected controls, 2) mild, and 3) severe shock rats, and 4) a separate group of rats pretreated with the neuronal reuptake blocker of norepinephrine, desmethylimipramine (DMI). The last group was employed to evaluate the singular effect of depressed reuptake in this experimental paradigm.

In the myocardium (Table VII) DMI had no effect on the level of endogenous norepinephrine, but DMI reduced the amount of ^{3}H-norepinephrine accumulated in the tissue by 95% ten minutes after

TABLE VI. EFFECT OF SYMPATHECTOMY WITH 6-HYDROXYDOPAMINE (6-OHDA) ON ENDOTOXIN-INDUCED LETHALITY

Hours After Endotoxin	% Lethality Control	% Lethality 6-OHDA
0	0 (0/27)#	0 (0/27)
1	0 (0/27)	0 (0/27)
2	7 (2/27)	0 (0/27)
3	7 (2/27)	0 (0/27)
4	19 (5/27)	7 (2/27)
5	22 (6/27)	7 (2/27)
6	26 (7/27)	* 7 (2/27)
7	26 (7/27)	* 7 (2/27)
8	30 (8/27)	* 11 (3/27)
9	52 (14/27)	** 15 (4/27)
10	56 (15/27)	** 15 (4/27)
11	56 (15/27)	** 19 (5/27)
12	56 (15/27)	** 19 (5/27)
.	.	.
.	.	.
.	.	.
24	56 (15/27)	** 26 (7/27)

* $p<0.05$, ** $p<0.01$ compared to control by Chi Square;
# numbers in parentheses indicate number of deaths / total initial number of animals;

Rats were treated with 6-OHDA 48 hours before endotoxin challenge. There were a total number of 27 rats in each group at time 0 hours.

TABLE VII. IN VIVO ^{3}H-NOREPINEPHRINE (^{3}H-NE) UPTAKE IN RAT MYOCARDIUM

Group	N	Plasma Glucose (mg/dl)	Norepinephrine (µg/gm)	^{3}H-NE (dpm/gm)
Control	7	91 ±3	0.80 ±0.05	11838 ±845
Desmethyl-imipramine	7	87 ±7	0.72 ±0.04	** 650 ±110
Mild Shock	15	95 ±5	** 0.66 ±0.01	13414 ±740
Severe Shock	7	** 23 ±3	** 0.48 ±0.06	17783 ±2904

Mean ± SEM

** $p<0.05$ compared to control by independent analysis of variance;

TABLE VIII. IN VIVO ^{3}H-NOREPINEPHRINE (^{3}H-NE) UPTAKE IN RAT SPLEEN

Group	N	Plasma Glucose (mg/dl)	Norepinephrine (μg/gm)	^{3}H-NE (dpm/gm)
Control	7	91 ±3	0.86 ±0.02	2309 ±224
Desmethyl-imipramine	7	87 ±7	0.94 ±0.09	** 366 ±48
Mild Shock	15	95 ±5	** 0.50 ±0.05	** 1522 ±238
Severe Shock	7	** 23 ±3	** 0.27 ±0.06	** 270 ±69

Mean ± SEM

** $p<0.05$ compared to control by independent analysis of variance;

intravenous injection of the labeled bioamine. In both mild and severely shocked endotoxic rats, ^{3}H-norepinephrine uptake was not changed from control. However, note that the relative depletion of endogenous norepinephrine is similar to the group of rats in which the depletion was demonstrated (Figure 9).

In the spleen (Table VIII), DMI pretreatment again had no effect on endogenous norepinephrine content, but it depressed the accumulation of injected ^{3}H-norepinephrine by 84%. Mild and severe shock produced depression of the endogenous norepinephrine content of the spleen. However, unlike the myocardium, during both mild and severe endotoxicosis, spleen uptake of circulating ^{3}H-norepinephrine was depressed. In mild shock, ^{3}H-norepinephrine content in spleens was reduced from a control value of 2309 $\pm$ 224 dpm/gm to 1522 $\pm$ 238 dpm/gm (34% reduction). Severe shock rats demonstrated an 88% decrease in ^{3}H-norepinephrine accumulation to 270 $\pm$ 69 dpm/gm.

2. IN VITRO ^{3}H-NOREPINEPHRINE UPTAKE

Because in vivo experiments suggested that reuptake depression would be most prominent in severe shock rats, in vitro analysis of norepinephrine uptake was performed in control and severe shock groups. The results of these experiments for heart and spleen slices are shown in Figures 18 through 21. Tissue slices were incubated for 0, 10, 20, or 30 minutes at 37° C in the presence of labeled and unlabeled norepinephrine. Total ^{3}H and ^{3}H-norepinephrine were measured.

Figure 18.

Total ^{3}H retention in myocardial slices of control and severely shocked rats incubated for the indicated time with ^{3}H-norepinephrine. Each bar represents the mean $\pm$ SEM for 6 preparations, each from a different rat. NS represents no significant difference for severe shock group versus control.

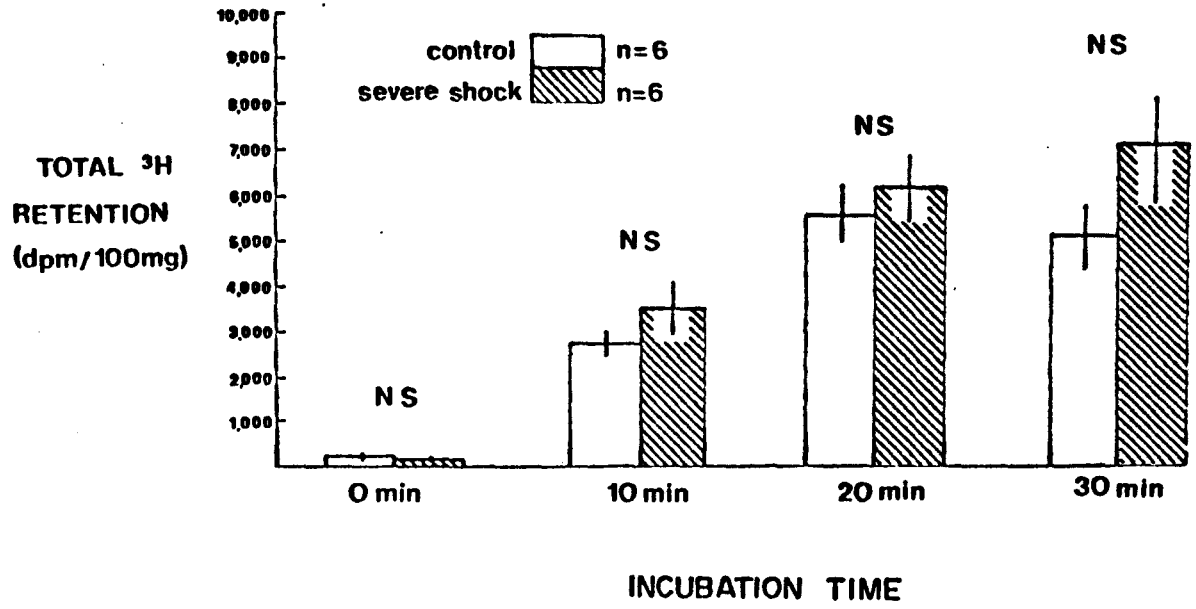

TOTAL ³H
RETENTION
(dpm/100mg)
10,000
9,000
8,000
7,000
6,000
5,000
4,000
3,000
2,000
1,000
control n=6
severe shock n=6
NS
NS
NS
NS
0 min
10 min
20 min
30 min
INCUBATION TIME

Figure 19.

Total norepinephrine retention for myocardial slice preparations of Figure 18 calculated from the ^{3}H-norepinephrine content of the tissue and the specific activity of the incubation media. Each bar represents the mean $\pm$ SEM for 6 preparations, each from a different rat. NS represents no significant difference for severe shock group versus control.

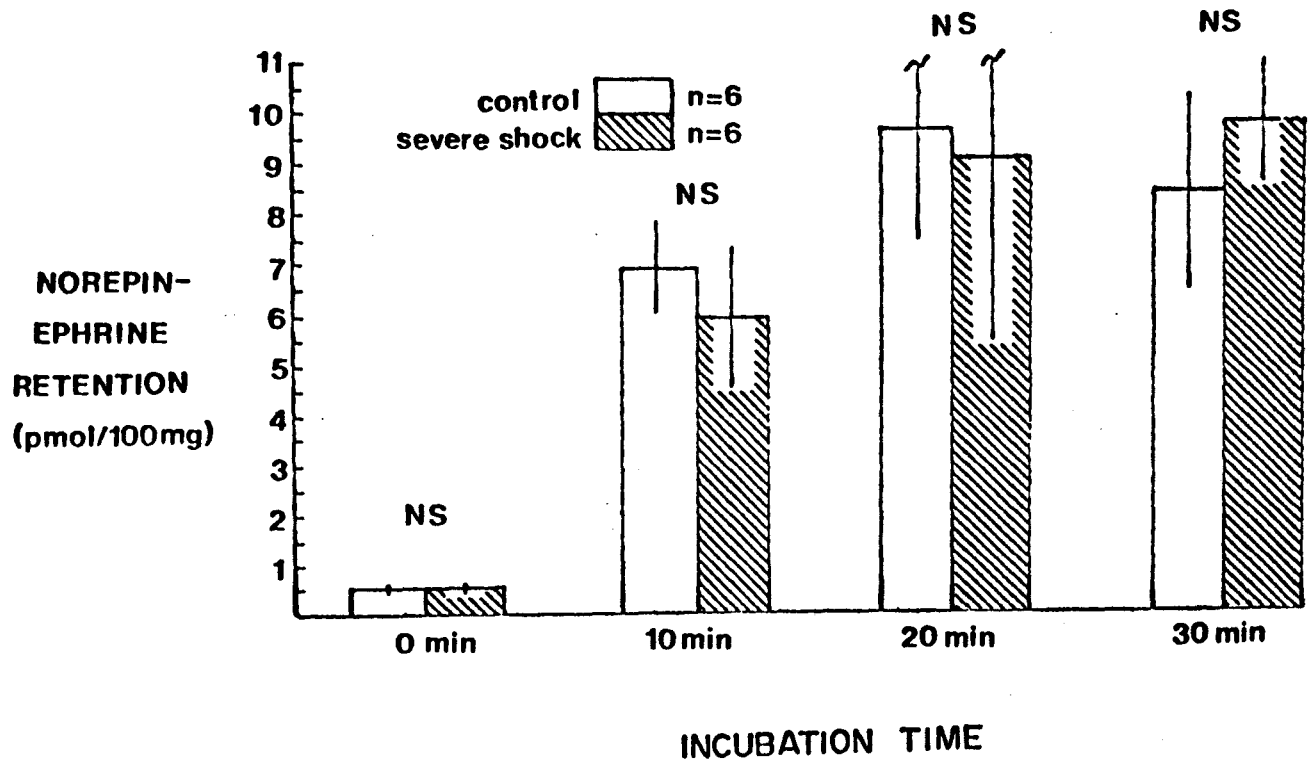

NS
NS
NS
control
severe shock
n=6
n=6
NOREPIN-
EPHRINE
RETENTION
(pmol/100mg)
11
10
9
8
7
6
5
4
3
2
1
0 min
10 min
20 min
30 min
INCUBATION TIME

Figure 20.

Total ^{3}H retention in splenic slices of control and severely shocked rats incubated for the indicated time with ^{3}H-norepinephrine. Each bar represents the mean $\pm$ SEM for 5 preparations, each from a different rat. NS represents no significant difference for severe shock group versus control.

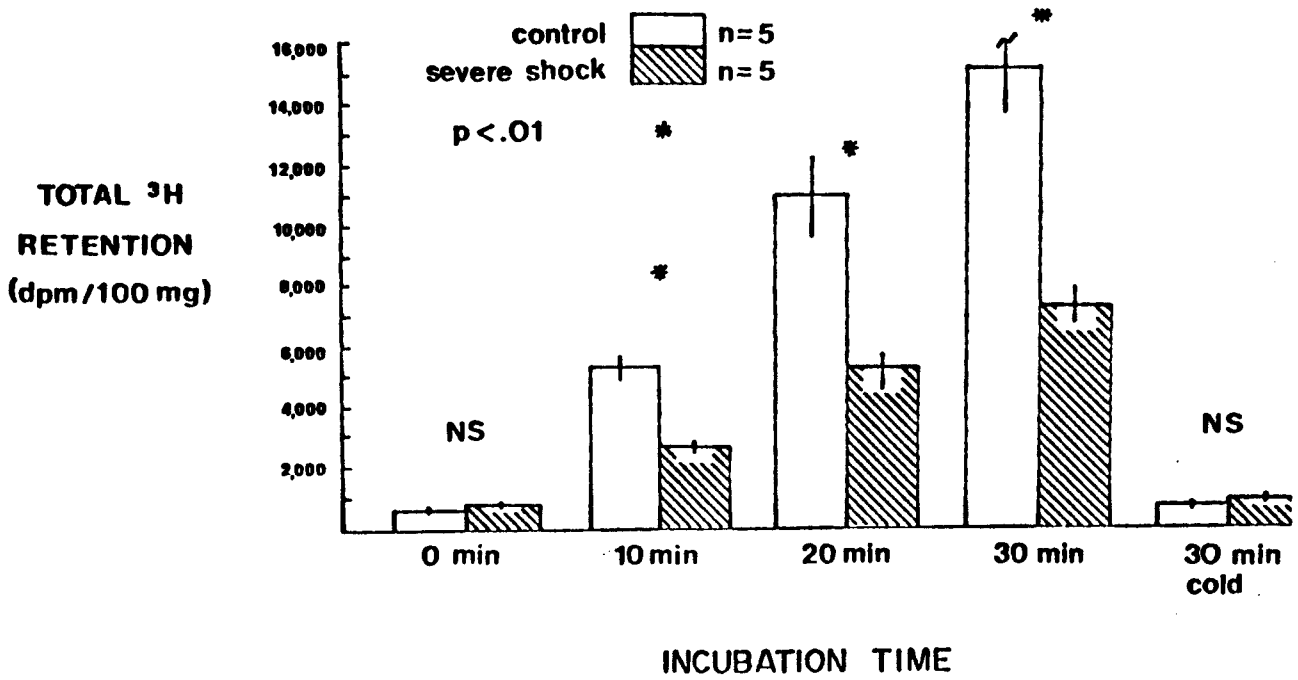
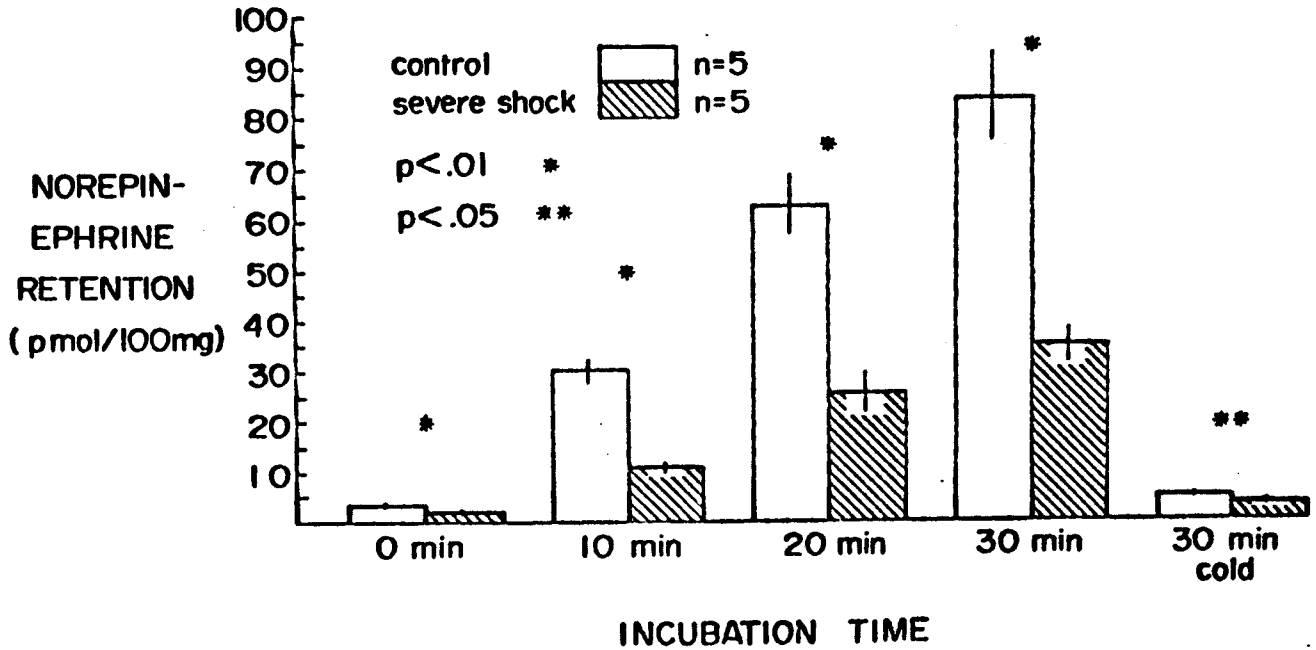

control
severe shock
n=5
n=5
p<.01
TOTAL ³H
RETENTION
(dpm/100 mg)
16,000
14,000
12,000
10,000
8,000
6,000
4,000
2,000
NS
NS
*
*
*
*
0 min
10 min
20 min
30 min
30 min
cold
INCUBATION TIME

Figure 21.

Total norepinephrine retention for splenic slice preparations of Figure 20 calculated from the ^{3}H-norepinephrine content of the tissue and the specific activity of the incubation media. Each bar represents the mean $\pm$ SEM for 5 preparations, each from a different rat.


NOREPIN-
EPHRINE
RETENTION
(pmol/100mg)
100
90
80
70
60
50
40
30
20
10
control
severe shock
n=5
n=5
p<.01 *
p<.05 **
0 min
10 min
20 min
30 min
30 min
cold
INCUBATION TIME

Norepinephrine retention (pmol/100 mg) was calculated as the quotient of the tissue ^{3}H-norepinephrine content (dpm/mg tissue) and the specific activity of the incubation media (dpm/pmol norepinephrine). In myocardial slices (Figure 18) at all times of incubation at 37° C there were no differences in total ^{3}H retention between control and severe shock rats. Similarly, total norepinephrine retention was the same in both groups of heart slices (Figure 19). In the spleen (Figures 20 and 21), total ^{3}H and total norepinephrine retention were severely reduced in tissue slices of severely shocked rats at all times of incubation at 37° C. The reduction was on the order of 50% at all times tested. The results of *in vitro* analyses are thus consistent with *in vivo* experiments, i.e. depressed reuptake of norepinephrine occurs in spleens, but not in hearts of endotoxic rats.

Because neuronal reuptake is temperature sensitive, incubations were also carried out for 30 minutes at 4° C. Cold incubation (Figures 20 and 21) effectively abolished all norepinephrine retention. Preliminary experiments with DMI in the incubation media indicated reuptake depression equal to cold incubation. Thus, the measured norepinephrine retention is dependent on neuronal reuptake and not non-neuronal reuptake or non-specific binding.

One profound consequence of shock is splanchnic congestion and accumulation of cellular debris in the spleen. Increase in tissue wet weight during shock would thus lower the measured norepinephrine accumulation values. To evaluate this possibility, both spleens and

hearts were removed from control and severely shocked animals, weighed, and then dried to constant weight. The results are shown in Table IX. There were no differences in wet weight, dry weight, or dry to wet weight ratios in hearts from control and shock animals. In the spleen, wet weight increased from 0.851 $\pm$ 0.038 gm to 1.013 $\pm$ 0.082 gm and dry weight increased from 0.195 $\pm$ 0.009 gm to 0.239 $\pm$ 0.023 gm in the endotoxic state. Both of these increases approached but did not reach significance ($p=0.10$ for both wet and dry weight differences). The splenic dry to wet weight ratio also did not change in the shock state (control: 0.229 $\pm$ 0.001; severe shock: 0.234 $\pm$ 0.004; $p=0.23$).

Although the weight changes in the spleen were not significant, the difference represents a 19% increase in wet weight and a 22% increase in dry weight in the severe shock rats. The parallel tendency for both spleen wet and dry weight to increase with no change in the dry to wet weight ratio suggests an increase in total cellular mass (most likely a congestion with red blood cells or cellular debris) of the spleen rather than a pure edema. The number of reuptake sites per unit of tissue wet weight will decrease if the spleen is infiltrated by cellular debris. Therefore, the measured amount of norepinephrine retention per 100 mg of tissue would be expected to decrease (in this case by 19%) simply due to an increase in cellular mass of the spleen. Therefore, the norepinephrine data for the spleen was corrected for changes in spleen wet weight of the endotoxic group (Figure 22). Even using this conservative modification, spleen slices from endotoxic rats retained significantly less norepinephrine than those from controls.

TABLE IX: MYOCARDIUM AND SPLEEN WEIGHTS IN CONTROL AND ENDOTOXIC RATS

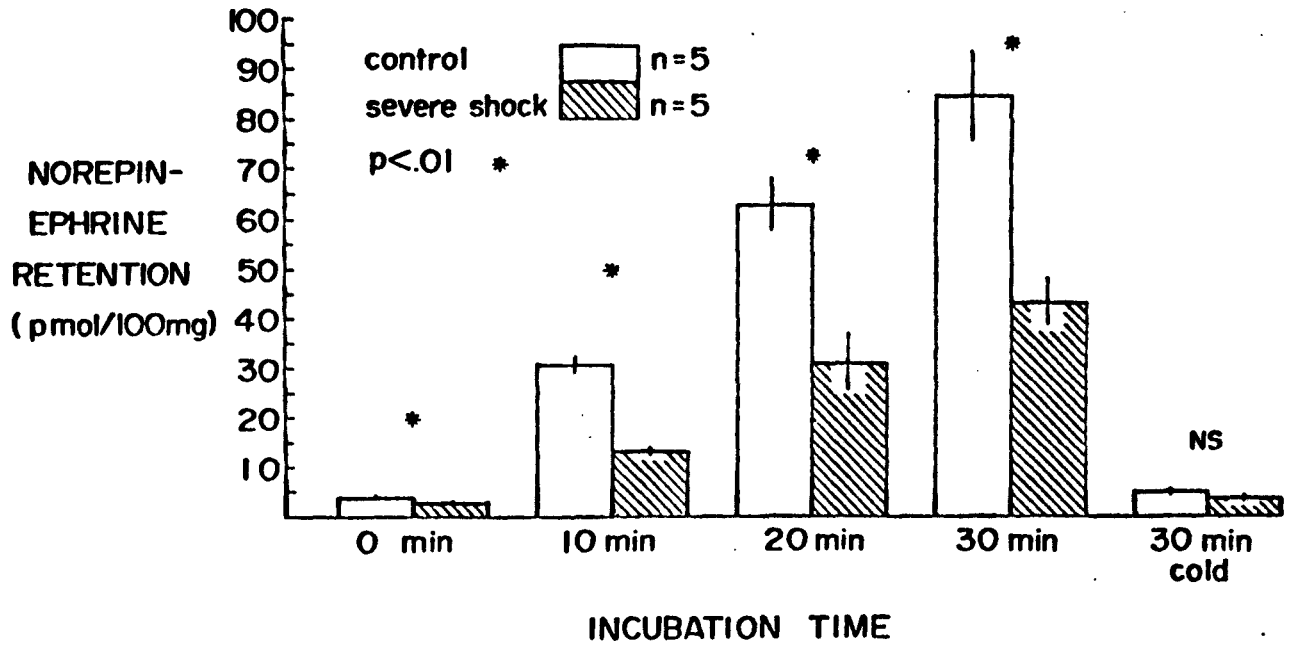
	CONTROL	MILD SHOCK	SEVERE SHOCK
N	8	8	7
Plasma Glucose (mg/dl)	110 ±4	* 82 ±5	* 35 ±5
MYOCARDIUM			
Wet Weight (gm)	1.491 ±0.035	1.485 ±0.036	1.429 ±0.088
Dry Weight (gm)	0.351 ±0.010	0.347 ±0.008	0.331 ±0.020
Dry/Wet Weight Ratio	0.236 ±0.003	0.234 ±0.002	0.234 ±0.003
SPLEEN			
Wet Weight (gm)	0.851 ±0.038	** 1.154 ±0.091	1.013 ±0.082
Dry Weight (gm)	0.195 ±0.009	** 0.261 ±0.023	0.239 ±0.023
Dry/Wet Weight Ratio	0.229 ±0.001	0.234 ±0.003	0.234 ±0.004

Mean ± SEM;

* $p<0.01$ vs control,
** $p<0.05$ vs control by independent analysis of variance;

Figure 22.

Total norepinephrine retention for spleen slice data of Figure 21 corrected for a 19% wet weight increase in spleens from severely shocked rats. Each bar represents the mean ± SEM for 5 preparations, each from a different rat. NS represents no significant difference for severe shock group versus control.


100
90
80
70
60
50
40
30
20
10
NOREPIN-
EPHRINE
RETENTION
(pmol/100mg)
control
n=5
severe shock
n=5
p<.01
*
*
*
*
*
NS
0 min
10 min
20 min
30 min
30 min
cold
INCUBATION TIME

3. INRACELLULAR DEGRADATION OF ^{3}H-NOREPINEPHRINE AFTER UPTAKE

Because the neuronal reuptake process (Uptake 1) is energy dependent and is compromised in the spleen, it is reasonable to suspect other energy sensitive processes to be affected in the spleen. After reuptake of norepinephrine, repackaging of the catecholamine into vesicles requires energy. One consequence of decreased rate of repackaging is an increase in intraneuronal degradation by the soluble enzyme, monoamine oxidase. The ratio of ^{3}H-norepinephrine to ^{3}H recovered by a tissue is indicative of the amount of norepinephrine taken up, repackaged, and conserved in its unmetabolized form. Figure 23 depicts the results of these calculations in spleen slices. At all times of incubation, tissues from shocked rats demonstrated significantly less ^{3}H retained in the form of ^{3}H-norepinephrine. Therefore, these data are consistent with the hypothesis that in endotoxic rats, norepinephrine degradation is accelerated in the nerve terminals of peripheral tissues.

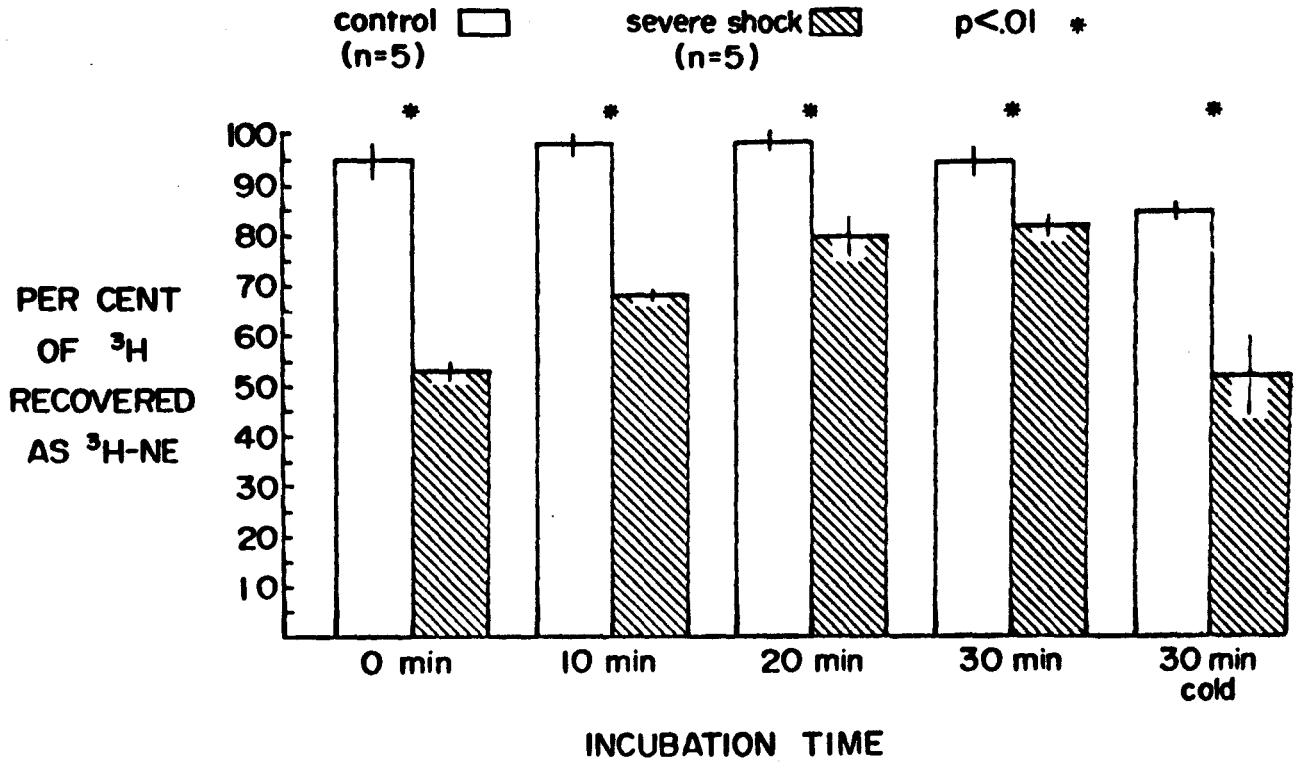
D. Measurement of Sympathetic Activity in Hearts and Spleens of Control and Endotoxic Rats

1. TISSUE NOREPINEPHRINE LEVELS IN CONTROL AND ENDOTOXIC RATS

Kinetic analysis of norepinephrine turnover by the ^{3}H-norepinephrine decay method is dependent on steady state tissue

Figure 23.

Per cent of radioactive label (^3H) recovered in its unmetabolized form (^3H-norepinephrine) in spleen slices of control and severely shocked rats coincubated with ^3H-norepinephrine for the indicated time. Each bar represents the mean $\pm$ SEM for 5 preparations, each from a different rat.


control
(n=5)
severe shock
(n=5)
p<.01
*
PER CENT
OF ³H
RECOVERED
AS ³H-NE
100
90
80
70
60
50
40
30
20
10
0 min
10 min
20 min
30 min
30 min
cold
INCUBATION TIME

levels of the bioamine throughout the 12 hour experiment (12,27). Table X illustrates the norepinephrine content in hearts and spleens of saline-injected control and endotoxic rats. Groups of rats were sacrificed at each of the indicated times after ^{3}H-norepinephrine injection. An independent analysis of variance was performed on each of the four groups of values. No statistical differences ($p>0.10$) in norepinephrine content exist between sampling times for any single organ experimental group. Thus, the reduced dose of endotoxin (2 mg/rat; 5.7-6.7 mg/kg) used in this series of experiments was sufficient to prevent the norepinephrine depletion of endotoxicosis. However, it is important to note that the stress of endotoxicosis in these particular experiments (LD 5%) is much less than in the general shock model (60 - 70%) used in the rest of the experiments.

2. NOREPINEPHRINE TURNOVER IN ENDOTOXICOSIS

Figure 24 illustrates the decay of myocardial ^{3}H-norepinephrine specific activity in control and endotoxic rats. Norepinephrine specific activity (dpm/ug norepinephrine) is plotted on a logarithmic scale. The slope of the specific activity versus time decay line for hearts from endotoxic rats (-0.047) was significantly increased ($p<0.001$) compared to the slope calculated for saline-injected control rats (-0.006). Figure 25 depicts specific activity decay lines for spleens of control and endotoxic rats. The slope of the line for the endotoxic group (-0.049) was markedly increased ($p<0.001$) over the control slope (-0.015). The constant level of endogenous norepinephrine

TABLE X. TISSUE LEVELS OF NOREPINEPHRINE IN HEARTS AND SPLEENS OF CONTROL AND ENDOTOXIC RATS

CONTROL		HOURS AFTER ^{3}H-NE INJECTION	ENDOTOXIN	
n	NE µg/g		n	NE µg/g
HEART				
5	0.70 ± 0.04	0.17	7	0.66 ± 0.03
6	0.72 ± 0.06	3.00	7	0.69 ± 0.04
6	0.69 ± 0.04	6.00	6	0.68 ± 0.06
6	0.67 ± 0.04	9.00	6	0.72 ± 0.05
6	0.66 ± 0.03	12.00	4	0.69 ± 0.04
SPLEEN				
5	0.96 ± 0.05	1.00	4	0.97 ± 0.10
7	1.01 ± 0.11	3.00	6	0.76 ± 0.06
7	0.90 ± 0.06	6.00	5	0.96 ± 0.07
6	0.93 ± 0.08	9.00	6	0.89 ± 0.09
3	0.76 ± 0.08	12.00	4	0.78 ± 0.01

Mean ± SEM; There are no statistical differences in norepinephrine content at different times after injection within any single group (independent analysis of variance).

Figure 24.

Decay of myocardial ^{3}H-norepinephrine (^{3}H-NE) specific activity in control and endotoxic rats. Rats were injected with ^{3}H-NE one minute before injection of either saline or endotoxin. Each point represents 4 to 7 rats. Slope of the decay line for endotoxic rats is significantly steeper than control ($p<0.001$).

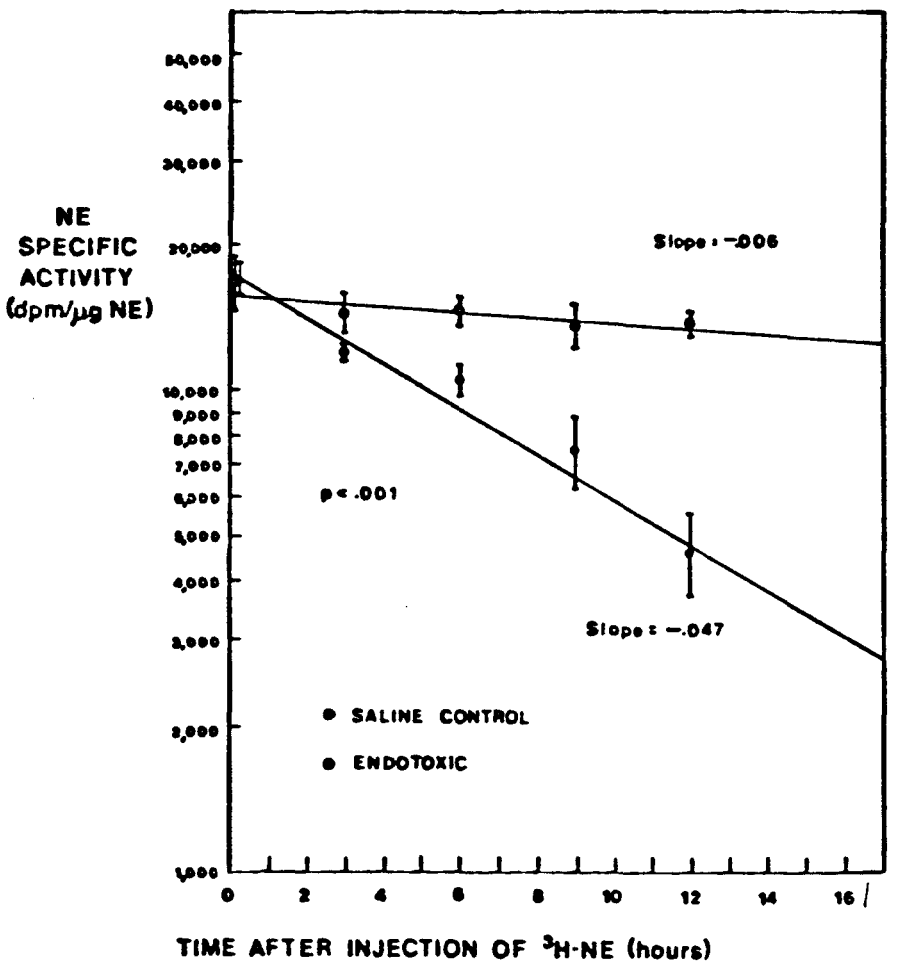
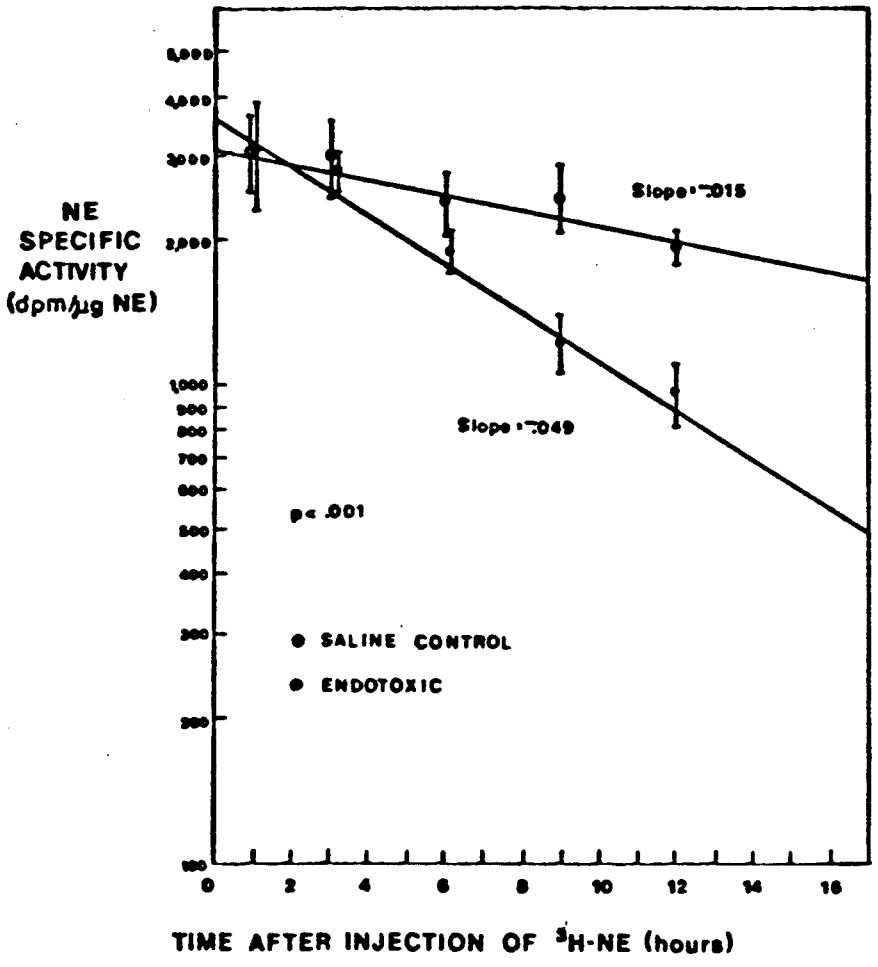

NE SPECIFIC ACTIVITY (dpm/µg NE)
Slope = -.006
p < .001
Slope = -.047
SALINE CONTROL
ENDOTOXIC
0
2
4
6
8
10
12
14
16
TIME AFTER INJECTION OF ³H-NE (hours)

Figure 25.

Decay of splenic ^{3}H-norepinephrine (^{3}H-NE) specific activity in control and endotoxic rats. Rats were injected with ^{3}H-NE one minute before injection of either saline or endotoxin. Each point represents 4 to 7 rats. Slope of the decay line for endotoxic rats is significantly steeper than control ($p<0.001$).


NE SPECIFIC ACTIVITY (dpm/µg NE)
Slope = .015
Slope = .049
p< .001
SALINE CONTROL
ENDOTOXIC
0
2
4
6
8
10
12
14
16
TIME AFTER INJECTION OF ^{3}H-NE (hours)

and increased rate of ^{3}H-norepinephrine disappearance from tissues of endotoxic rats indicate increased turnover of norepinephrine in the endotoxic state.

The kinetic turnover data for hearts and spleens is summarized in Table XI. In hearts the mean norepinephrine content for all samples comprising a single decay curve was unchanged in the endotoxic group compared to control. Rate constants were calculated from the slopes and application of first order kinetics. The rate of cardiac norepinephrine turnover (the product of the rate constant and the endogenous norepinephrine content) increased more than 700% in the endotoxic group (0.075 $\mu g \times gm^{-1} \times hr^{-1}$) compared to controls (0.009 $\mu g \times gm^{-1} \times hr^{-1}$). Similar results occurred in the spleen. Endogenous norepinephrine content of all endotoxic spleens (0.87 $\pm$ 0.04 μg/gm) was not significantly different ($p=0.24$) than control spleens (0.93 $\pm$ 0.04 μg/gm). Endotoxin treatment more than tripled the rate constant of decay from 0.035 hr^{-1} in the control state to 0.114 hr^{-1}. Thus, the turnover rate of norepinephrine in the spleen increased 200% in the endotoxic state (control: 0.033 $\mu g \times gm^{-1} \times hr^{-1}$; endotoxin: 0.099 $\mu g \times gm^{-1} \times hr^{-1}$).

3. EFFECT OF DESMETHYLIMIPRAMINE ON NOREPINEPHRINE TURNOVER

Increased norepinephrine turnover rates in endotoxicosis indicate increased sympathetic tone. However, the experiments just reported demonstrated that spleens but not hearts from endotoxic rats displayed depressed norepinephrine reuptake when measured in both in

TABLE XI. NOREPINEPHRINE (NE) TURNOVER IN HEARTS AND SPLEENS OF CONTROL AND ENDOTOXIC RATS

TREATMENT	n	NE µg/g	SLOPE	RATE CONSTANT hr^{-1}	TURNOVER RATE µg/(gxhr)
HEART					
Control	29	0.69 ±0.02	-0.006 ±0.004	0.013	0.009
Endotoxin	30	0.69 ±0.02	-0.047* ±0.006	0.108	0.075
SPLEEN					
Control	28	0.93 ±0.04	-0.015 ±0.008	0.035	0.033
Endotoxin	25	0.87 ±0.04	-0.049* ±0.007	0.114	0.099

Mean ± SEM;
* $p<0.001$ compared to control using the standard error of the sample regression.

vivo and in vitro situations. It is possible that the depressed splenic norepinephrine reuptake of endotoxic rats may contribute to increased rate of transmitter loss from the terminal (both labeled and unlabeled) and be measured as increased turnover rate. Therefore, this hypothesis was tested by measuring norepinephrine turnovers in control rats pretreated with the neuronal norepinephrine reuptake inhibitor, DMI. Turnovers in both hearts and spleens were examined to determine if Uptake 1 had similar effects on sympathetic activity in both organs.

Table XII illustrates the norepinephrine content in hearts and spleens of DMI-treated rats. The critical organ for turnover measurement is the spleen, since reuptake has been shown to be depressed in endotoxic spleens. At each time point splenic norepinephrine content was found to not be statistically different than any other time point. The same holds true for myocardial norepinephrine content. The control group of rats is the same as in the endotoxic turnover experiments. The cardiac and splenic norepinephrine content for the control rats is depicted in Table X.

The specific activity decay lines for hearts and spleens of control and DMI-treated rats are depicted in Figures 26 and 27, respectively. Although the initial specific activity is not the same, the slopes for specific activity decay are not statistically different from one another in hearts of control (-0.006) and DMI-treated (-0.004) rats ($p>0.015$; Figure 26). Figure 27 illustrates that the slopes of the splenic specific activity decay lines were the same (0.015 hr^{-1}) for

TABLE XII. TISSUE LEVELS OF NOREPINEPHRINE (NE) IN HEARTS AND SPLEENS OF DESMETHYLIMIPRAMINE-TREATED RATS

NE µg/gm	HOURS AFTER ^{3}H-NE INJECTION	n
HEART		
0.83 ± .05	1.0	6
0.73 ± .06	3.0	6
0.77 ± .05	6.0	6
0.72 ± .05	9.0	6
0.70 ± .08	12.0	5
SPLEEN		
0.85 ± .10	1.0	4
0.78 ± .04	3.0	5
0.75 ± .09	6.0	6
0.72 ± .05	9.0	6
0.81 ± .08	12.0	5

Mean ± SEM; Rats were injected with desmethylimipramine 10 minutes after ^{3}H-NE injection. There are no statistical differences in norepinephrine content at different times after injection within any single group (independent analysis of variance).

Figure 26.

Decay of myocardial ^{3}H-norepinephrine (^{3}H-NE) specific activity in control and desmethylimipramine-treated rats. Rats were injected with ^{3}H-NE ten minutes before injection of desmethylimipramine. Each point represents 4 to 7 rats. Slope of the decay line for desmethylimipramine-treated rats is no different from control ($p>0.015$).

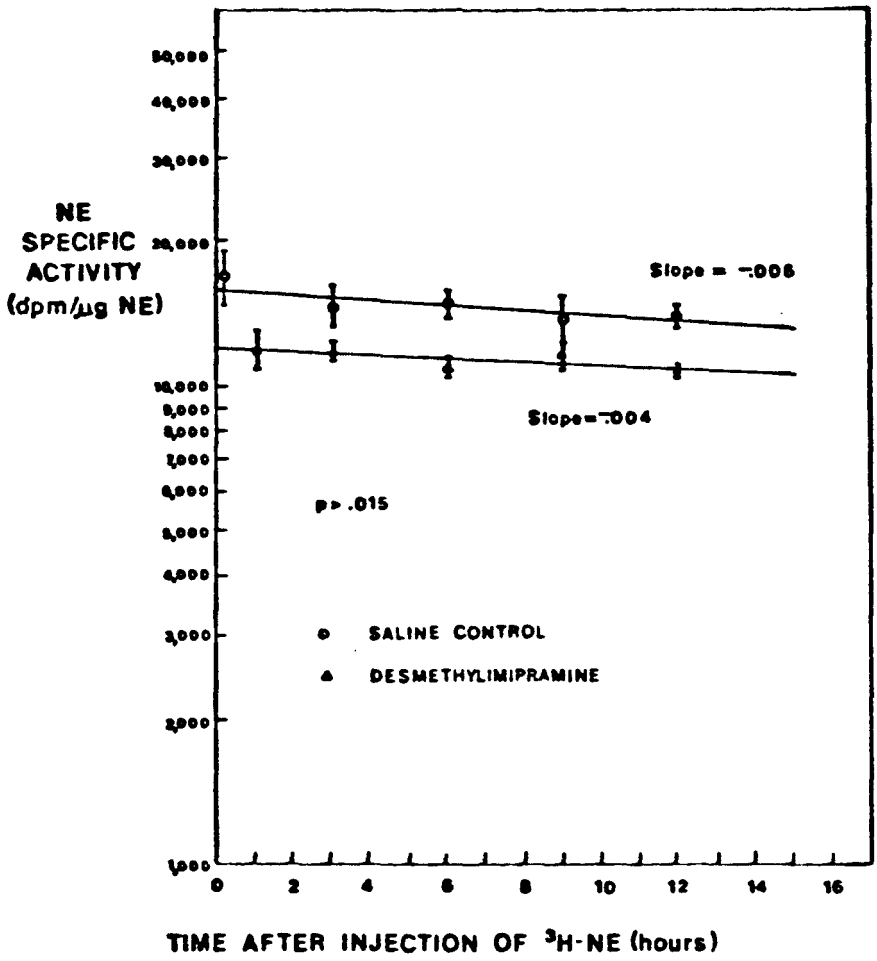
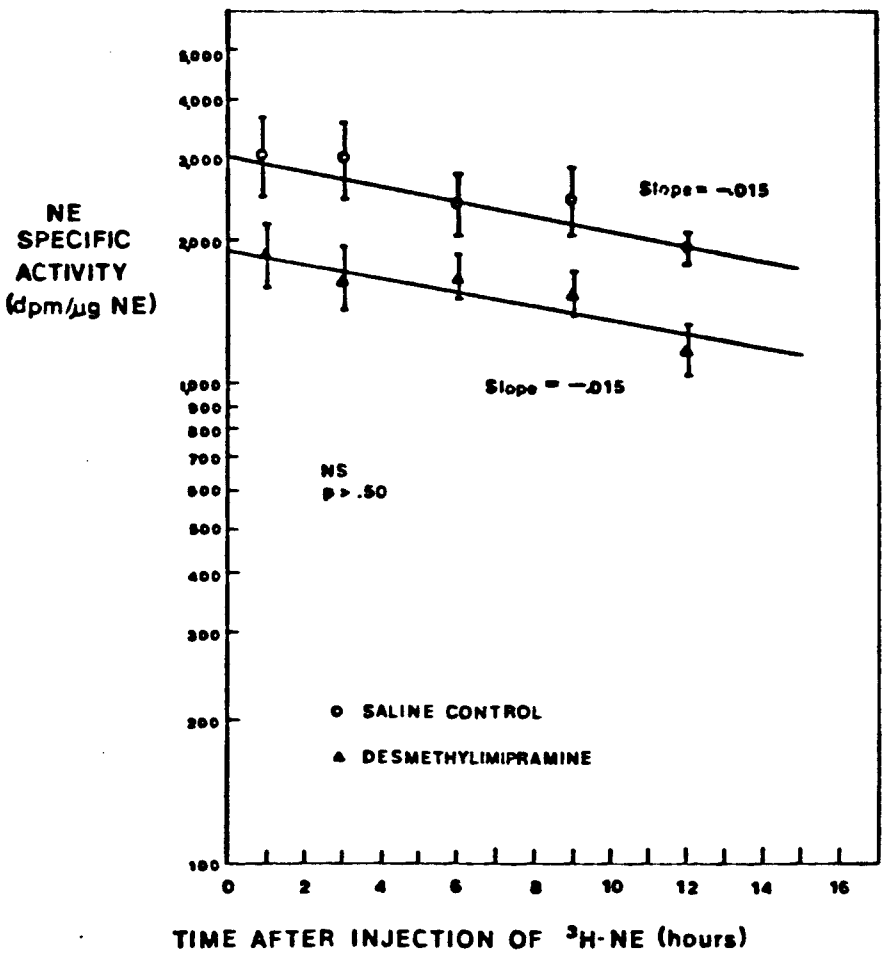

NE
SPECIFIC
ACTIVITY
(dpm/µg NE)
Slope = -.008
Slope=-.004
p>.015
SALINE CONTROL
DESMETHYLIMIPRAMINE
0
2
4
6
8
10
12
14
16
TIME AFTER INJECTION OF ³H-NE (hours)

Figure 27.

Decay of splenic ^{3}H-norepinephrine (^{3}H-NE) specific activity in control and desmethylimipramine-treated rats. Rats were injected with ^{3}H-NE ten minutes before injection of desmethylimipramine. Each point represents 4 to 7 rats. Slope of the decay line for desmethylimipramine-treated rats is no different from control ($p>0.50$).


NE SPECIFIC ACTIVITY (dpm/µg NE)
5,000
4,000
3,000
2,000
1,000
900
800
700
600
500
400
300
200
100
Slope = −.015
Slope = −.015
NS
p > .50
SALINE CONTROL
DESMETHYLIMIPRAMINE
0
2
4
6
8
10
12
14
16
TIME AFTER INJECTION OF ³H-NE (hours)

both control and DMI-treated rats. Although the specific activity at 0 hours after ^{3}H-norepinephrine was greater in the control group, the fractional loss of tracer from both groups was exactly the same.

Table XIII summarizes the turnover data for control versus DMI-treated rats. In hearts, DMI pretreatment increased the endogenous level of norepinephrine. However, the rate constant of decay in hearts of DMI-treated rats was reduced compared to control. The turnover rates in both groups of rats were similar (0.009 μg/(gxhr) in controls, 0.006 μg/(gxhr) for DMI treatment) and extremely lower than the turnover rate for endotoxic rats (0.075 μg/(gxhr). In spleens, DMI treatment reduced endogenous levels of norepinephrine slightly, but significantly. The fraction of norepinephrine turned over per hour was the same in both groups. Therefore, the splenic turnover rate of DMI-treated rats was slightly lower (0.028 $\mu g x g m^{-1} x h r^{-1}$) than in controls (0.033 $\mu g x g m^{-1} x h r^{-1}$). Again, as in the heart, note that turnovers in spleens of control and DMI-treated rats were dramatically lower than in spleens of endotoxic rats (0.099 μg/(gxhr)).

TABLE XIII. NOREPINEPHRINE (NE) TURNOVER IN HEARTS AND SPLEENS OF CONTROL AND DESMETHYLIMIPRAMINE-TREATED RATS

TREATMENT	n	NE µg/g	SLOPE	RATE CONSTANT hr^{-1}	TURNOVER RATE µg/(gxhr)
HEART					
Control	29	0.69 ±0.02	-0.006 ±0.004	0.013	0.009
Desmethyl-imipramine	29	0.75* ±0.03	-0.004* ±0.003	0.008	0.006
SPLEEN					
Control	28	0.93 ±0.04	-0.015 ±0.008	0.035	0.033
Desmethyl-imipramine	26	0.78* ±0.03	-0.015 ±0.029	0.036	0.028

Mean ± SEM;
* $p<0.001$ compared to control using the standard error of the sample regression.

CHAPTER VI

DISCUSSION

The present experiments have focused on one aspect of sympathetic dysfunction during endotoxin shock: depletion of norepinephrine from sympathetic nerve terminals. These studies can be categorized into four areas: 1) demonstration of the phenomenon, 2) characterization of the depletion, 3) assessment of norepinephrine reuptake, and 4) measurement of sympathetic activity during endotoxin shock. Each of these areas will be discussed separately.

A. Demonstration of Norepinephrine Depletion during Endotoxin Shock

Fundamental to any series of experiments is an appropriate, reproducible model. In the present studies an endotoxic rat model was employed: intravenous injection of *Salmonella enteritidis* endotoxin at a dose (14 to 17 mg/kg) to generate a 60%-80% lethality. The albino rat has been widely used in endotoxic studies, primarily for metabolic, immune, and *in vitro* preparation experiments. The inbred genetic line of the laboratory rat minimizes animal to animal variations, and the size and cost per animal facilitates experimental repetition. Larger animal models, such as the dog or primate, are better suited for hemodynamic and cardiovascular experimentation - the so-called "pump and pipe" investigations. The area of present interest, norepinephrine

depletion from peripheral tissues, is particularly well-suited to a small animal model such as the rat. The rat is large enough to obtain adequately-sized tissues for analysis, and small enough to be economically feasible to generate an adequate number of samples. The terminal nature of the experiments and the sampling technique enable the studies to be performed in the absence of anesthesia. Previous endotoxic research using rats has generated a solid data base of information with respect to many metabolic, immunological, and even hemodynamic responses to endotoxin administration. Finally, although endotoxin-induced norepinephrine depletion has been previously demonstrated in the rat (120), the characteristics and mechanisms involved have not been adequately defined. Thus, the rat is an appropriate model for study of norepinephrine depletion and sympathetic dysfunction during endotoxicosis.

Initial experiments demonstrated norepinephrine depletion from hearts and spleens of endotoxic rats (Table III). The depletion was shown to be correlated with the terminal plasma glucose of the rats (Figures 9-12). This finding was consistent throughout the course of all the experiments. Previous work has shown that initially plasma glucose rises and then falls to hypoglycemic levels during endotoxicosis (45). Using this criteria, the norepinephrine depletion was shown to be associated with the severity of hypoglycemia, and hence the severity of the shock state. These results are consistent with the study of Pohorecky *et al.* (120) who observed that norepinephrine depletion from peripheral tissues in the endotoxic rat was a dose- and time-dependent

phenomenon. According to the protocol, rats were sacrificed one to five hours after endotoxin administration. Because animals progress through the shock state at different rates, the present model of sacrificing animals at 5 hours after endotoxin or near death sampled animals at all stages of shock. A qualitative observation was that animals surviving to the 5 hour limit were found to be of the mild shock group (plasma glucose greater than 40 mg/dl). Rats that neared death before the 5 hour period (and were sacrificed at that time) were usually of the severe shock group (plasma glucose less than 40 mg/dl). On this basis, the norepinephrine depletion could be considered to be inversely related to the time of endotoxicosis, and hence contrary to the results of the Pohorecky study. However, the discrepancy is resolved because the sampling procedure was different in the two studies. The present protocol allowed sacrifice of animals before five hours only if they were near death; the Pohorecky study selected animals only on the basis of time after endotoxin injection. If "time- and dose- dependence" is interpreted as the severity of shock, the results of both studies are in agreement.

Tissue epinephrine content was also measured in the present experiments. In many cases epinephrine data was not reported because of the limitations of the measurement. According to Crout (29), quantitative significance should only be ascribed to epinephrine values when they exceed 5% of the calculated norepinephrine content. Phenylethanolamine-N-methyl transferase (PNMT), the enzyme responsible for conversion of norepinephrine to epinephrine, has been found

primarily in the adrenal medulla (8) and to a small extent in the central nervous system (5,22,119). Low levels of epinephrine-forming activity have even been demonstrated in hearts of rabbits although the physiologic significance of it remains questionable (5). Therefore, epinephrine is not readily synthesized in non-adrenal peripheral tissues, although they generally contain small amounts of it. The origin of peripheral tissue epinephrine is most likely from circulating epinephrine that is recovered by the neuronal uptake process, Uptake 1 (76). Thus, the amount of epinephrine in a peripheral tissue is dependent upon the synthesis of epinephrine in the tissue, the circulating concentration of epinephrine in the blood, the blood flow to the tissue, the rate of neuronal uptake, and the extent of degradation of the amine inside the nerve terminal. Therefore, interpretation of peripheral tissue epinephrine content must be made with the above considerations in mind.

Cardiac epinephrine content increased during endotoxicosis (Table III). However, it is important to note that only 5 of 15 epinephrine values of control samples were used in the analysis while 21 of 21 endotoxic samples were included. The necessity to delete epinephrine values that were less than 5% of the corresponding norepinephrine values may have introduced a sampling error by having selected for data with abnormally high epinephrine to norepinephrine ratios. Therefore, saline control epinephrine content may be skewed to the high end of normal. This effect would minimize observed increases in epinephrine content during endotoxicosis. A significant increase was

noted, however, during shock and is considered a real change, although the quantitative significance of the control data must be qualified. Similarly, increased epinephrine content in peripheral non-adrenal tissue has been reported previously in hemorrhagic models (23,52,55). During endotoxin shock in the dog, a tendency toward increased epinephrine content in hearts, livers, and spleens has been demonstrated, but the results were not statistically different from contols (164). In light of the evidence for elevated plasma catecholamines during endotoxicosis, it is likely that circulating epinephrine was taken up by cardiac sympathetic nerve endings and subsequently stored there. The present experiments that demonstrated uncompromised neuronal reuptake in the myocardium also support this hypothesis.

B. Characterization of Norepinephrine Depletion

One consistent finding in the initial experimental series was the relationship between norepinephrine content and plasma glucose during endotoxicosis (see Figures 11 and 12). Classic views support the thesis that activation of the sympathoadrenal system during fasting or hypoglycemic states plays a role in maintaining metabolic substrate homeostasis (15,48,94). Another view has been championed recently by Landsberg and Young who demonstrated decreased norepinephrine turnovers in various peripheral organs during fasting and increased turnovers in the same organs during feeding and overfeeding (3,89,163). Adrenal medullary function (assessed by plasma epinephrine concentrations),

however, was shown to more closely approximate results expected by traditional theories (90,162). Despite the conflicting theories, it was reasonable to question whether the norepinephrine depletion associated with endotoxic shock was solely a function of decreased plasma glucose concentration. The singular role of hypoglycemia on heart and spleen norepinephrine content was assessed in a separate model of insulin hypoglycemia. Tissue samples were collected 90 minutes after intravenous injection of various doses of regular insulin. This particular regimen was developed to mimic the variable fall in plasma glucose of endotoxic rats. Additionally, because most of the hypoglycemic endotoxic rats were sacrificed from 3 to 5 hours post-injection, and because rats first experience a hyperglycemic phase before hypoglycemia, a 90 minute falling phase in plasma glucose (in the insulin group) is a reasonable parallel to the endotoxic state. The data indicated that insulin hypoglycemic rats did not display the norepinephrine depletion characteristic of endotoxicosis (Table IV). In similar fashion, regression analysis of tissue norepinephrine versus plasma glucose for insulin hypoglycemic rats failed to reveal a significant correlation (Figures 14 and 15). Lastly, neither the mild nor severe insulin hypoglycemic group displayed cardiac or splenic norepinephrine content significantly lower than saline controls. The results obtained do not preclude norepinephrine depletion in other, more severe (either lower plasma glucose or longer maintenance of hypoglycemia) models of insulin hypoglycemia. Thus, the conclusion of the experiments is that, in the model of insulin hypoglycemia employed, norepinephrine metabolism in peripheral tissues was not adversely

affected. Revealing experiments to be pursued in this area are: 1) tissue norepinephrine measurement during prolonged hypoglycemia to test the effect of the severity of the stress, and 2) measurement of norepinephrine turnovers in a prolonged model of insulin hypoglycemia (perhaps using longer acting lente and ultra-lente insulins). The latter experiments would reveal whether conditions for norepinephrine depletion were present (increased sympathetic activity) and would also provide a different situation (high insulin and low plasma glucose) for investigation of the Landsberg and Young hypothesis (when feeding or fasting induced physiologic parallel changes in plasma glucose and insulin).

Cardiac epinephrine content was also measured during insulin hypoglycemia. During hypoglycemia the epinephrine to norepinephrine ratio was generally high enough so that 37 of 41 samples met the criteria for epinephrine quantitation. Mean epinephrine content of the myocardium was increased during insulin hypoglycemia and was found to be inversely correlated with the plasma glucose concentration. These results are consistent with the interpretation that adrenal medullary secretion of epinephrine into the circulation was increased during the hypoglycemia, augmenting circulating levels of catecholamines, and thereby increasing uptake of amines into peripheral sympathetic nerve endings.

Fine and associates demonstrated in the dog that abdominal denervation protected against the lethality and splanchnic

histopathologic changes associated with endotoxic shock (114,165). The denervation also prevented the norepinephine depletion of the abdominal organs (165). Their conclusion that norepinephrine depletion was dependent upon neural drive was confirmed in the present experiments: Treatment of rats with the ganglionic blocker, chlorisondamine, prevented norepinephrine depletion in the heart during endotoxicosis (Figure 17). Because nerve section or blockade, as well as ganglionic blockade eliminated norepinephrine depletion, endotoxin probably did not directly cause release of norepinephrine. If endotoxin or its mediators directly elicited release of norepinephrine, the depletion would have occurred in the absence of neural activity.

Experiments other than the denervation studies of Fine have implicated the sympathetic nervous system in the pathogenesis of endotoxic shock. Abundant literature has been cited that documents the beneficial effects of alpha adrenergic blockade during endotoxicosis (10,57,58,96). One report also demonstrated that beta adrenergic blockade sensitized rats to endotoxic hypoglycemia and lethality. Based on the literature two questions become apparent: 1) Do interventions that protect against endotoxic shock (e.g. alpha adrenergic blockade) also protect against norepinephrine depletion, and vice versa (i.e. the effects of beta antagonist sensitization on tissue norepinephrine content)? 2) Does prior depletion of norepinephrine stores and hence decreased adrenergic stimulation by peripheral sympathectomy protect against endotoxic shock? Both questions were investigated.

Filkins has reported (46) that intraperitoneal pretreatment of rats with propranolol, a nonselective beta adrenergic antagonist, sensitized rats to lethality associated with endotoxic shock and that the animals also displayed compromised glucose homeostatic capability (depressed hepatic gluconeogenesis and hypoglycemia). Opposite results were found after alpha adrenergic blockade pretreatment with phentolamine. A similar model was employed in the present studies, although the dose of endotoxin administered to beta-blocked rats was higher (5 mg/300 gm rat) than in the previous study (3 mg/300 gm rat). Results confirmed the change in lethality with adrenergic blockade: all alpha adrenergic blockade rats survived the 5 hour protocol and all beta adrenergic blocked rats died within 30 minutes of endotoxin injection (mean time to death was 18 $\pm$ 1 minutes). Phentolamine pretreated rats had higher plasma glucose levels than rats administered endotoxin alone. However, no statistical decreases in plasma glucose of propranolol-treated rats was evident. This may be explained by the increased endotoxin dose employed in the propranolol rats. Death may have been caused by acutely fulminating cardiovascular or pulmonary changes (early systemic hypotension and pulmonary hypertension are common complications of endotoxicosis) that were not compensated for by a myocardium unresponsive to beta adrenergic stimulation. Thus, the late metabolic changes were not yet evident at the time of death (evidenced by fairly normal plasma glucose levels). In similar fashion, no depletion of tissue norepinephrine was seen in propranolol-treated rats. Again, the time to death may have been too short to observe any

changes. However, in phentolamine rats, cardiac and splenic norepinephrine content was greater at 5 hours than rats treated with endotoxin alone. It can be concluded that phentolamine protection during endotoxicosis is also associated with a tissue norpinephrine sparing effect. It is impossible, however, to determine whether, in the alpha-blocked rat, the improved glucose homeostasis, improved perfusion of various organs, or some direct effect of phentolamine to decrease sympathetic drive was responsible for the improved tissue norepinephrine levels.

As reviewed earlier, various forms of total peripheral sympathectomy have been investigated during endotoxin shock (2,9,58,99). Chemical sympathectomy with either guanethidine, bretylium tosylate, or 6-hydroxydopamine, was not shown to have a protective effect in newborn rats (2). However, sympathectomy with 6-hydroxydopamine did protect against lethality and fibrin deposition in rabbits (9). Reserpine pretreatment also significantly depleted tissue norepinephrine content and protected against endotoxin lethality in rabbits and mice (58). Finally, epidural spinal block sensitized dogs to endotoxic lethality (99). Despite the contradictory results, experiments in sympathectomized rats were performed for the following reasons: 1) The studies on newborn rats were conducted so early after birth that normal sympathetic reflexes in the control, non-sympathectomized rats may not yet have been developed enough to affect the shock state (54). 2) Studies that utilized spinal epidural anesthesia were simply not specific for ablation of only the sympathetic nervous system. All

descending spinal pathways were interrupted, causing stress and trauma independent of the endotoxic insult. 3) The phentolamine protection and propranolol sensitization experiments of this study suggested an intriguing concept. Chemical sympathectomy with 6-hydroxydopamine eliminates peripheral norepinephrine stores but leaves the adrenal medulla intact (containing an epinephrine/norepinephrine mixture) (146). In a very nonspecific way, sympathectomy eliminates much of the alpha adrenergic dominant norepinephrine and leaves much of the beta adrenergic epinephrine intact. Thus, sympathectomy, while drastically reducing sympathetic tone, also to some extent is weighted toward minimizing "alpha" tone and not affecting "beta" tone - a functional alpha blockade with minimal beta blockade. Chemical sympathectomy in the present model with 6-hydroxydopamine significantly decreased endotoxic lethality from 6 hours after endotoxin through the end of the 24 hour protocol. Post-mortem examination revealed qualitatively that mesenteric lesions were less severe in the sympathectomized rats. The lethality data is consistent with similar experiments of Bolton and Atuk (9) in the rabbit, and of Gourzis, Hollenberg, and Nickerson in the rabbit and mouse (58). These data indicated that this form of blunted sympathetic activity was also protective in the endotoxic rat. Further evaluation of metabolic parameters in the sympathectomized rat model may be important to elucidate any protective effects on glucose or fat metabolism.

C. Norepinephrine Reuptake in Endotoxic Shock

The experiments described thus far, have shown norepinephrine depletion to be associated with the severity of shock as assessed by plasma glucose concentrations, and to be dependent on neural drive. As Figure 1 illustrates, a constant tissue content of norepinephrine is dependent on an equilibrium between processes that increase the pool size (biosynthesis and neuronal reuptake) and that decrease it (sympathetic activity and intraneuronal degradation). The depletion of norepinephrine from tissues during endotoxic shock must result from the disturbance of one or more of the above described processes. Because the depletion is dependent on release of norepinephrine, and because reuptake is responsible for conservation of transmitter present in the synaptic cleft, decreased reuptake may contribute to norepinephrine depletion from nerve terminals. Indeed, Bhagat and Friedman (6) have stressed the importance of neuronal reuptake in maintaining pool size during conditions of high sympathetic tone. Additional previous observations during endotoxic shock are consistent with this hypothesis. Depressed reuptake would result in more release of catecholamines into the general circulation, a finding well-documented by increased concentrations of circulating catecholamines in endotoxicosis. Because reuptake also is the main mechanism for termination of transmitter action, a decrease in this process would intensify sympathetic-mediated responses. Increased vascular sensitivity to epinephrine has been shown after endotoxin administration (58,167), although conflicting results

have been reported (104). However, the demonstration that vasodilators protected against endotoxic shock (10,57,58,96) indicated that adrenergic vasoconstriction of the peripheral circulation was high during shock. Thus, adequate evidence has implicated depressed neuronal reuptake of norepinephrine in the norepinephrine depletion of endotoxin shock.

The present experiments were designed to evaluate whether depression of neuronal reuptake of norepinephrine occurred during endotoxic shock. Initial experiments assessed norepinephrine uptake by measuring the amount of injected ^{3}H-norepinephrine taken up and stored by hearts and spleens. Previous studies have been conducted that utilized similar protocols in the rat (120) and dog (123). These experiments have already been reviewed in detail, but the differences in methodology from the present work need to be stressed. Pohorecky et al. (120) described increased norepinephrine reuptake measured in vivo early after endotoxin administration. The methods were similar to those used in the present study. However, the measurement was made only one hour after endotoxin injection - shorter than in the present experiments. Also, in the Pohorecky study the endotoxin was administered intraperitoneally and not intravenously. The demonstration of increased reuptake may be due to the model employed or to elevated plasma catecholamine levels that are associated with endotoxicosis. Observations have been made that indicate increased norepinephrine reuptake during periods of high sympathetic activity when substrate concentrations are high (17,50). The Rao et al. investigation (123)

also assessed norepinephrine reuptake *in vivo* by measuring the incorporation of radiolabeled norepinephrine into endogenous stores. However, the time between tracer injection and tissue sampling was too long (150 minutes) to ascribe the results to solely norepinephrne reuptake.

Both of the aforementioned studies (120,123), as well as the present experiments, utilized the alumina chromatography method for isolation of catecholamines before counting the samples for ^{3}H-norepinephrine. However, the alumina chromatography technique does not eliminate the deaminated metabolite, 3,4-dihydroxyphenylglycol (DOPEG) (59). DOPEG has been shown to be a substantial fraction of the ^{3}H released from various tissues after loading of the tissues with ^{3}H-norepinephrine (59,91,144,145). Therefore, it is reasonable that in any of the studies in question, DOPEG may have, in part, been measured in the eluate after alumina chromatography. However, the interest is in the catecholamines retained within the tissue and not released from it. The following studies have reported on experiments with tissues previously loaded with ^{3}H-norepinephrine that have measured norepinephrine and its metabolites that were retained in, as well as released from the tissues (19,59,145).

Graefe *et al.* (59) measured the radioactivity spontaneously released from and retained in isolated, rat vas deferens preparations perfused with ^{3}H-norepinephrine. Norepinephrine and its metabolites were separated by alumina chromatography followed by a Dowex 50Wx4

column (to separate norepinephrine from DOPEG). The spontaneous outflow of ^{3}H-catechols from the preparation was measured two hours after the end of incubation with ^{3}H-norepinephrine. Results indicated that 70% of ^{3}H released spontaneously from the isolated, perfused organ was in the form of DOPEG. However, at the same time after incubation, ^{3}H-norepinephrine accounted for over 95% of the total radioactivity retained within the tissue. In similar fashion, Tarlov and Langer (145) investigated the effect of field stimulation on the release of labeled norepinephrine from rat and guinea pig atria. Norepinephrine and its metabolites were separated by paper chromatography. Results indicated that 90% of the radioactivity released spontaneously from atria was associated with norepinephrine metabolites - primarily deaminated byproducts (including DOPEG). Field stimulation increased the release of radioactivity and also the fraction of ^{3}H recovered as ^{3}H-norepinephrine (60% norepinephrine and 40% deaminated metabolites). Analysis of the radioactivity retained within atria 150 minutes after the end of incubation with ^{3}H-norepinephrine demonstrated that more than 90% of the radioactivity corresponded to the norepinephrine chromatographic peak. Similar results have been demonstrated in isolated canine hearts (19).

Therefore, the present and cited studies which assessed norepinephrine reuptake during endotoxicosis with ^{3}H-norepinephrine and alumina column chromatography have assurance from related studies that the measured radioactivity was associated with norepinephrine rather than its metabolites. It should also be noted that even if the

recovered ^{3}H-norepinephrine was metabolized prior to measurement, the total radioactivity would still be an accurate measure of uptake since the metabolism occurred after reuptake. Had metabolism of norepinephrine occurred before reuptake, the byproducts would have been o-methylated extraneuronally by COMT. Uptake 1 will not transport amines with methoxyl groups present on the aromatic ring (76). The results of the present in vivo experiments (Tables VII and VIII) indicated that norepinephrine reuptake was depressed in spleens, but not in hearts of endotoxic rats. The depression of reuptake in the spleen was directly related to the severity of the shock state. Experiments in this study have already demonstrated that norepinephrine depletion from the heart and spleen was also related to the severity of the shock state. Although the present experiments indicated that the spleen functionally takes up less norepinephrine in endotoxicosis, the results may be reasonably interpreted in two ways: 1) reuptake was depressed in nerve endings of the spleen, and 2) during endoxicosis, blood flow was shunted away from the spleen, and hence less delivery of labeled norepinephrine to the spleen occurred.

Support for the latter hypothesis is present in existing literature. Measurements of blood flow distribution in various endotoxic models indicated that while the per cent of cardiac output to the myocardium remained unaltered or elevated, the proportion to splanchnic organs was severely limited (42,83,159,160). In the unanesthetized monkey, Wyler et al. (159) used radiolabeled microspheres to measure cardiac output distribution both acutely (at the end of a 40

minute infusion period) and 24 hours after E. Coli endotoxin infusion. Acutely, all vascular beds demonstrated some degree of vasodilatation except the spleen, in which vascular resistance tripled, and blood flow decreased from 134 to 24 ml/(min x 100 gm tissue). At the same time, coronary resistance fell, and total coronary flow actually increased to 142 ml/(min x 100 gm) compared to saline-injected, time-matched, controls (98 ml/(min x 100 gm)). Twenty-four hours after endotoxin infusion, results from heart and spleen were essentially the same: directional changes in vascular resistance compared to time-matched controls were the same, but the magnitude of the responses were reduced. The same group of investigators (160) studied the distribution of cardiac output in unanesthetized endotoxic rabbits using similar techniques. Measurements were made 0, 60, and 150 minutes after endotoxin injection. Vascular resistance increased most dramatically in the spleen, kidneys, and skin of endotoxic rabbits compared to time-matched controls. Vasoconstriction in the coronary circulation was minimal during endotoxicosis and often was no different from time-matched control values.

Blood flow measurement using radiolabeled microspheres has also been applied to rodent models. Ferguson et al. (42) measured regional blood flow in the unanesthetized endotoxic guinea pig. The percentage of cardiac output to the spleen was drastically reduced at both one and three hours after endtoxin injection. The coronary circulation received a higher proportion of the cardiac output during endotoxicosis. However, since cardiac output fell, both organs had lower than control

flow after endotoxin, but the flow deprivation to the spleen was much greater than to the myocardium. In the rat, Keeler et al. (83) used two slow infusions of endotoxin (10 mg/kg over 4 hours; 2.5 to 3 mg/kg infused over a period of four days) to more closely simulate a septic state. The acute (4 hour) infusion was lethal to 64% of the rats; chronic infusion (4 days) resulted in no lethality. Under anesthesia, cardiac output and its distribuion were measured using radioactive microspheres. Cardiac output was reduced in both acute and chronic models of endotoxicosis, although the reduction was greatest in the acute situation. The per cent of cardiac output to the myocardium remained unchanged in both acute and chronic endotoxicosis compared to control. Percentage distribution of cardiac output to liver, gastrointestinal tract, and skin fell in both models of endotoxemia. Although spleen flows were not measured, hepatic and gastrointestinal data may be examined as representative of abdominal organ responses.

Thus, distribution of cardiac output during endotoxicosis has been shown to consistently decrease in the spleen, but may not change or actually increase in the heart. Labeled norepinephrine uptake from the circulation is especially sensitive to blood flow distribution to various organs. In the present experiments norepinephrine reuptake assessment was dependent on circulatory distribution; thus the decreased amount of labeled norepinephrine found in the spleen may have been the result of decreased distribution of flow to that region. Because flow distribution changes little in the myocardium, results in that organ may

be more representative of the actual uptake process rather than a function of delivery.

Therefore, experiments were undertaken to study norepinephrine reuptake in a model where delivery of the labeled bioamine was not flow-limited. Incubation of tissue slices *in vitro* effectively controlled substrate availability to the reuptake sites. It also ensured that both control and endotoxic preparations were oxygenated to the same extent. Results of *in vitro* experiments indicated similar findings as the *in vivo* studies: norepinephrine reuptake and retention was depressed in spleen slices but not myocardial slices from endotoxic rats (Figures 19 and 21). Because spleen wet weight increased during endotoxicosis and results were based on a wet weight basis, the norepinephrine retention values were corrected to eliminate the possible error. After correction, spleen slices from endotoxic rats still retained far less norepinephrine than spleen slices from controls. The differential depression of norepinephrine uptake between organs raises a question regarding the mechanism responsible for it. If endotoxin or its mediators were responsible for reuptake inhibition, the effect should have been evident in both hearts and spleens. However, because neuronal reuptake of norepinephrine is energy dependent (76), and because blood flow is severely reduced to the spleen (but not the heart) during endotoxicosis (42,83,159,160), reuptake depression in the spleen may have been caused by flow and hence energy deprivation secondary to endotoxin administration. The ischemia and acidosis in the spleen was probably more severe in the latter stages of shock, which is also the

time when norepinephrine uptake was most severely limited in the in vivo experiments.

Another energy dependent process associated with monoamine metabolism is the uptake of norepinephrine from neuronal cytoplasm into vesicles. Because neuronal reuptake has been compromised, it is reasonable to assume that the repackaging process has also been affected. Neuronal reuptake without concomitant revesicularization results in degradation of the norepinephrine by monoamine oxidase. Thus, if repackaging was depressed, much of the norepinephrine taken up by the reuptake process would be reduced to metabolites. The data in Figure 23 illustrate that relatively more of the norepinephrine taken up by spleen slices of control rats remained unmetabolized than in the spleens of severely shocked rats. Thus, these results suggest that increased intraneuronal degradation occurred in spleens of endotoxic rats, which may contribute to the norepinephrine depletion that occurs in that organ. Experiments on endotoxic guinea pigs (7) also provided evidence consistent with this hypothesis.

D. Norepinephrine Turnovers during Endotoxicosis

Norepinephrine turnovers accurately reflect the level of sympathetic nerve activity (12,26,27) in various organs. Assessment may be made by any of the following methods: 1) Measurement of radiolabeled norepinephrine formed after infusion (111,133) or rapid injection (133) of radiolabeled tyrosine yields an accurate estimate of norepinephrine

synthesis and can be used to calculate turnover rates. 2) When norepinephrine synthesis is inhibited with alpha-methyltyrosine, endogenous norepinephrine levels decline at a rate proportional to the concentration of remaining amine. A graph of endogenous norepinephrine content versus time mathematically describes a first order relationship, and is used to calculate a rate constant of decay and a turnover rate (12,109). 3) Injection of labeled norepinephrine is taken up by sympathetic neurons and released along with endogenous norepinephrine. Under conditions of constant endogenous levels of norepinephrine the relationship of specific activity of tissue norepinephrine versus time describes a first order decay (105,26).

Each technique has its positive aspects as well as its drawbacks. The labeled tyrosine methodology yields synthesis rates from single determinations while the other techniques necessitate use of groups of animals. However, the tyrosine method also relies on either an indwelling cannula or an acutely anesthetized animal model to facilitate infusion of tyrosine. The unanesthetized model would be of choice, but the possibility of stress and infection from prior surgery may complicate the endotoxin model. Additionally, methodology for analysis of tyrosine would be required. The alpha-methyltyrosine technique is reliable and can be performed in groups of unanesthetized animals, but the technique measures the decline in norepinephrine content over time. Because endotoxin administration alone can deplete norepinephrine from tissues, the first order decay of norepinephrine content induced by alpha-methyltyrosine injection may not occur during

endotoxicosis. The ^{3}H-norepinephrine technique also depends on groups of animals to calculate a turnover rate. The method relies on constant levels of endogenous norepinephrine which may be a drawback during endotoxin shock. This methodology, however, does not by itself disturb the constancy of the endogenous norepinephrine content (which is not possible after alpha-methyltyrosine injection). However, the ^{3}H-norepinephrine technique was the procedure of choice to estimate sympathetic activity during endotoxicosis for the following reasons: 1) Low doses of endotoxin eliminated norepinephrine depletion as well as lethality. Furthermore, the constancy of the endogenous norepinephrine content was measured to ensure proper kinetic analysis. 2) Animals were studied in a non-invasive, unanesthetized model. 3) Sympathetic activity was assessed over a long period of time, rather than acutely during any short phase during endtoxicosis.

Because the depletion of norepinephrine from peripheral organs was shown to be time- and dose-dependent (120), low dose endotoxin treatment (lethality was 5% at 24 hours) was used to minimize the depletion of norepinephrine. Preliminary experiments demonstrated that this regimen did not deplete norepinephrine from hearts or spleens. On occasion, however, tissue content of norepinephrine was drastically depressed in samples obtained 12 hours after endotoxin. These rats were probably more sensitive to endotoxin than others and were excluded from the study. Basal cardiac turnover rates in control animals are similar to those found in the literature, but tend to be on the slower end of those published (3,89,24). However, the experimental paradigm used in

the present experiments may be responsible for this. The rats were housed singly for three days before the start of the turnover studies. Isolation may have an effect to lower sympathetic activity. Additionally, the rats were fasted for eighteen hours before and also during the turnover measurement. Fasting has been shown to decrease norepinephrine turnovers to various peripheral organs (89,90,163). Comparison of the present turnover rates with those of other studies that have also measured them in a fasting state show good similarity (3,89).

Endotoxin treatment accelerated the turnover of norepinephrine in hearts and spleens of rats compared to controls (Figs. 24 and 25, Table XI). Thus, the average sympathetic activity to hearts and spleens of endotoxic rats was increased over the 12 hours after endotoxin injection. This conclusion is consistent with previous studies that used radiolabeled norepinephrine injections, although none of the investigations were designed to assess turnover rates quantitatively (16,120,123). While the results of the present experiments are also consistent with some electrophysiological results during endotoxicosis (61), they do not agree with the work of others (86,130). Halinen demonstrated increased cardiac sympathetic efferent nerve activity early after endotoxin injection in dogs (61). Koyama et al. (86) and Santiesteban et al. (130) demontrated decreased preganglionic splanchnic nerve activity in the endotoxic cat. Major differences in models and experimental design exist between the electrophysiological and turnover studies. The use of an invasive, anesthetized cat or dog model is

difficult to compare to the unanesthetized rat. Furthermore, the turnovers estimated nerve activity over a twelve hour period after endotoxin; nerve recording was performed for brief periods of time early after endotoxin. Additionally, the differences in species and endotoxin doses could easily have induced differential changes in sympathetic activity.

Experiments of the present study demonstrated that norepinephrine reuptake was reduced in spleens of endotoxic rats in severe shock. Even though the low dose endotoxin injection for turnover measurement induced a modest lethality (5%) compared to the fundamental shock model (70% lethality), similar reuptake depression may have occurred. Depressed reuptake leads to a reduction of released transmitter that is recovered by the nerve terminal. It is possible that during turnover measurement in the spleen, reduced recovery of norepinephrine and ^{3}H-norepinephrine caused an accelerated decay rate of norepinephrine specific activity, thus falsely indicating increased sympathetic nerve activity to that organ. This possibility was tested by treating control rats with a potent neuronal reuptake inhibitor, desmethylimipramine, before norepinephrine turnover measurement. The results indicated that the singular effect of decreased norepinephrine reuptake did not alter the fraction of norepinephrine released per hour (Figures 26 and 27, Table XIII). Therefore, it is concluded that even if splenic norepinephrine reuptake was depressed in the present endotoxic model, it did not contribute to the observed increased rate of norepinephrine turnover.

The increased norepinephrine turnovers in hearts and spleens may have been the result of increased central drive of sympathetic nerves. However, electrophysiological experiments on isolated nerve-muscle preparations suggested that endotoxin affected information processing at the presynaptic site (115,117,118). One hypothesis was that endotoxin increased the amount of transmitter released per nerve impulse. If this indeed occurred in the *in situ* situation, norepinephrine turnovers may have increased without a concomitent increase in nerve activity. Alternatively, increased release per stimulus coupled with even a modest increase in sympathetic activity could result in greatly increased norepinephrine turnovers. The effect of increased norepinephrine release per stimulus would effectively accelerate the loss of norepinephrine from the existing pool (both labeled and unlabeled) and increase the replacement with newly formed unlabeled norepinephrine. Regardless of the mechanism for the increased turnovers measured in hearts and spleens of endotoxic rats, the turnover rates may have been magnified under conditions of higher endotoxin administration and contribute to the norepinephrine depletion that occurs in lethal endotoxic shock.

E. Implications and Conclusions

The role of the sympathetic nervous system in endotoxic shock is multi-faceted, and many of its aspects remain to be elucidated. Demonstrations of increased circulating concentrations and depleted

tissue content of catecholamines suggested that sympathetic activity was high during endotoxic shock. While a classic view of the sympathetic nervous system is that it aids in the maintenance of homeostasis during stressful or "fight or flight" situations, the sympathetic system may be a double edged sword in shock. Early during shock the sympathetic system may maintain blood presure and enhance metabolic substrate availability. However, the utilization of these compensatory mechanisms may exhaust both circulatory and metabolic reserve leading to the irreversible phase of shock. Presumably the vasoactive properties of catecholamines cause prolonged flow deficits to multiple organ systems, most notably the splanchnic region, to maintain perfusion of more flow sensitive areas such as heart and brain. Additionally, the metabolic effects of catecholamines may alter substrate availability as well as metabolism. Although the sympathetic nervous system may tide an animal through the early phases of shock, it may ultimately exacerbate the situation later in the form of ischemic organs and metabolic derangements. Many past reports as well as data presented here demonstrated the beneficial effects of reduced sympathetic stimulation during endotoxicosis.

Norepinephrine depletion from peripheral tissues is one manifestation of sympathetic dysfunction during endotoxicosis that warrants further study. In light of increased plasma concentrations of catecholamines during shock, the depletion most likely represents a massive transfer of catecholamines from nerve terminals to synaptic spaces and finally to the general circulation. Thus, prevention of the

depletion would also prevent hyperactivation of adrenergic receptors. As discussed earlier, the major candidates for the mechanism of norepinephrine depletion were considered to be increased sympathetic activity and depressed neuronal reuptake. These hypotheses are consistent with the existing literature and also fit within the conceptual framework of sympathetic exacerbation of the shock syndrome. Increased sympathetic drive and decreased neuronal reuptake both result in greater release of catecholamines from nerve terminals. This is in contrast to hypotheses of singular inhibition of norepinephrine synthesis or increased intraneuronal degradation which would result in depletion, but not increased liberation of transmitter.

Elucidation of the mechanism and subsequent prevention of the increased sympathetic activity as well as interventions to maintain splenic reuptake of norepinephrine may well be future goals to formulate protective interventions in not only endotoxic shock, but also its clinical counterpart, sepsis.

SUMMARY

Activation of the sympathetic nervous system during endotoxic shock has been demonstrated by a variety of methods: elevation of plasma catecholamines, depletion of tissue norepinephrine, altered disposition of administered radiolabeled catecholamines, and increased nerve activity measured directly by electrophysiological techniques. However, these studies have been largely indirect and/or contradictory in nature. Furthermore, a large volume of literature has implicated the sympathetic nervous system - either in a primary or secondary role - in the pathogenesis of endotoxic shock. Experiments were designed to investigate the following aspects of sympathetic nervous system dysfunction during endotoxicosis in the white laboratory rat: 1) demonstrate and further characterize norepinephrine depletion from heart and spleen, 2) assess norepinephrine reuptake both *in vivo* and *in vitro* in hearts and spleens by measuring the amount of ^{3}H-norepinephrine incorporated into those organs, and 3) quantitatively estimate the degree of sympathetic activity in both hearts and spleens of endotoxic rats by measurement of norepinephrine turnovers by the ^{3}H-norepinephrine decay technique.

Initial experiments demonstrated that the depletion from hearts and spleens was related to the severity of the shock state as evidenced by the significant negative correlation of norepinephrine content to the

plasma glucose concentration. A separate group of rats treated with insulin demonstrated no depletion and suggested that the depletion with endotoxin was not caused by the singular effect of low plasma glucose. Ganglionic blockade with chlorisondamine prevented the depletion from the heart (spleen was not tested) which provided evidence that the depletion was neurally-mediated. Alpha adrenergic blockade (phentolamine) before endotoxin injection significantly increased survival time, maintained normal plasma glucose levels, and resulted in higher cardiac and splenic norepinephrine content than in non-treated endotoxic rats. Beta adrenergic blockade (propranolol) before endotoxin injection significantly decreased survival time and exacerbated the hypoglycemia of endotoxicosis, but did not result in significant changes in cardiac or splenic norepinephrine content compared to non-treated endotoxic rats. Depletion of peripheral tissue norepinephrine content before endotoxin administration (sympathectomy with 6-hydroxydopamine) significantly decreased the lethality associated with endotoxin administration.

Uptake of ^{3}H-norepinephrine from the circulation was depressed in spleens, but not hearts of endotoxic rats (uptake depression was greater in spleens of severely shocked rats). Because blood flow distribution has been shown to change during endotoxicosis, *in vitro* experiments were repeated on hearts and spleens of control and severely shocked rats. Results of *in vitro* incubation of tissue slices with ^{3}H-norepinephrine were similar to *in vivo* results: uptake of labeled norepinephrine was depressed in spleens, but not hearts of endotoxic

rats compared to control. Additionally, less of the labeled norepinephrine was retained in its unmetabolized form in endotoxic spleens. The results indicated that reuptake depression may be a factor in the depletion of norepinephrine from spleens, but not hearts of endotoxic rats. Furthermore, uptake and degradation may also play a role in splenic norepinephrine depletion.

Norepinephrine turnovers were increased in both hearts and spleens of rats administered a low dose of endotoxin (LD 5%). Turnovers were also measured in a group of rats injected with the norepinephrine reuptake blocker, desmethylimipramine to assess the singular role of reuptake inhibition on turnover rate. Comparison of turnover rates in control and desmethylimipramine-treated rats demonstrated no differences. Results indicated quantitatively that sympathetic activity was increased to hearts and spleen of endotoxic rats.

It is concluded that norepinephrine depletion from hearts and spleens may be mediated by increased sympathetic activity to those organs. Additionally, decreased neuronal reuptake of norepinephrine may play a role in the norepinephrine depletion in the spleen, but not in the heart. Both mechanisms would result in increased stimulation of adrenergic receptors and contribute to the hypersympathetic state associated with the pathogenesis of endotoxic shock.

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

11/9/82
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