# THE ROLE OF CALCIUM IN SUPERSENSITIVITY INDUCED BY COCAINE



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To

my loving and understanding wife

Arlene

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## ABSTRACT

An alternative to the uptake hypothesis as the sole cause of supersensitivity due to cocaine has been proposed. Calcium is required for the contraction of smooth muscle and its utilization in contraction is a post-receptor event. Evidence is presented that cocaine may owe at least part of its effect in potentiating noradrenaline and histamine to changes in calcium utilization.

Cocaine was shown to alter the utilization of membrane or intracellular calcium in the contraction of the isolated spleen strip by noradrenaline. Noradrenaline caused a small contraction of the isolated spleen strip in the absence of extracellular calcium and reduced tissue calcium. This small noradrenaline contraction was greatly potentiated by cocaine. The repeated administration of Na-EDTA antagonized the cocaine potentiation of noradrenaline, and greatly reduced the magnitude of subsequent responses to noradrenaline and cocaine. It is suggested that cocaine potentiates noradrenaline by making more bound calcium available for release, and that EDTA antagonizes the potentiation by chelating this calcium once it is released and diffuses out of the cell along its concentration gradient.

Diazoxide also antagonized the cocaine potentiation of the contraction to noradrenaline in a calcium-free solution, but did not antagonize the contraction to noradrenaline. It is suggested that diazoxide prevents cocaine from making bound calcium more available for release by noradrenaline.

Cocaine potentiated the contraction of the spleen strip to strontium in the absence of extracellular calcium. It is presumed that the contraction to strontium, like that of noradrenaline, is

due to the release of bound calcium and that the potentiation by cocaine is due to an increased availability of this calcium.

The changes in the utilization of bound calcium for contraction by cocaine were not due to an increase in the noradrenaline concentration at the receptor as the result of either the release of endogenous noradrenaline or the blockade of uptake of exogenous noradrenaline. Cocaine potentiated the contraction to noradrenaline in a calcium-free solution where the endogenous noradrenaline stores were depleted by reserpine. Cocaine increased the maximum noradrenaline contraction in the absence of extracellular calcium. Cocaine also potentiated the responses to noradrenaline in the spleen strip where neuronal uptake had already been blocked by DMI.

Cocaine also altered the utilization of extracellular calcium by noradrenaline for contraction of the spleen strip. Less extracellular calcium was necessary for an equivalent noradrenaline contraction in the presence of cocaine than in the control. These results suggest that cocaine either increased the membrane permeability to calcium, caused release of more bound calcium, or sensitized the contractile elements so that they utilized calcium more efficiently.

The contraction of the spleen strip by histamine was greatly reduced in the absence of extracellular calcium, and was not potentiated by cocaine. However, noradrenaline potentiated the contraction to histamine in a calcium-free solution. The failure of cocaine to potentiate histamine was attributed to the inability of histamine to release bound calcium, and the noradrenaline potentiation attributed to the release of bound calcium which was then utilized by histamine for contraction.

Cocaine, however, did alter the utilization of extracellular calcium for contraction by histamine, but a larger concentration of cocaine was required than for noradrenaline. Less extracellular calcium was required for an equivalent histamine contraction in the presence of cocaine than in the control. These results suggest that cocaine increases the utilization of extracellular calcium by facilitating its access to the contractile elements or altering the contractile elements so that they respond more efficiently to calcium.

Desmethylimipramine (DMI) blocked the uptake of noradrenaline in the isolated reserpine-treated spleen strip, but DMI was not as effective as cocaine in this respect. A larger concentration of DMI than cocaine was required to block the uptake of noradrenaline. Together DMI and cocaine caused a greater reduction in noradrenaline uptake than did DMI alone, but not cocaine alone.

Depending upon its concentration DMI either potentiated or antagonized the responses to noradrenaline in the spleen strip. Small concentrations of DMI potentiated the noradrenaline contraction of the spleen strip in the presence of normal calcium, but not in a calcium-free solution. These small DMI concentrations did not alter the amount of extracellular calcium required for contraction of the spleen strip by noradrenaline. Large concentrations of DMI non-competitively antagonized the contraction of the spleen strip by noradrenaline in the presence of normal calcium. However, these large concentrations of DMI did not antagonize the response to noradrenaline in the absence of extracellular calcium. It is suggested that the potentiating effect of DMI in the presence of extracellular calcium is due to blockade of neuronal uptake, while the antagonism is due to the prevention

of the utilization of extracellular calcium by noradrenaline for contraction.

Procaine which has been shown to cause efflux of $^{45}Ca$ in skeletal muscle, did not potentiate the contraction of the isolated spleen strip by noradrenaline in either a solution containing the normal calcium content or in a calcium-free solution. Procaine also failed to alter the utilization of extracellular calcium required for contraction of the spleen strip by noradrenaline. It was also found that procaine did not modify the potentiation of noradrenaline by cocaine in a calcium-free solution.

TABLE OF CONTENTS

## INDEX TO FIGURES

INDEX TO TABLES

# INTRODUCTION

## A. SUPERSENSITIVITY CAUSED BY COCAINE

The ability of cocaine to potentiate the effect of adrenaline on the blood pressure of the rabbit and on pupillary dilatation of the cat was first observed by Fröhlich and Loewi (1910). Cocaine was also found to potentiate the responses to sympathetic nerve stimulation, both in the pressor response in spinal, adrenalectomized cats (Rosenblueth, 1931), and in the contraction of the cat's nictitating membrane (Rosenblueth & Cannon, 1931; Rosenblueth & Rioch, 1933). These authors postulated that the sensitizing action of cocaine could occur at the effector cell by acting on the contractile mechanism, by diminishing the rate of destruction of catecholamines, or by increasing the combination of catecholamines with substances in the muscle.

Studies on the potentiating action of cocaine early became complicated by several puzzling observations of the effects of cocaine on responses to tyramine, a compound chemically related to adrenaline and possessing many of its actions. Tainter & Chang (1927) reported that cocaine antagonized the pressor action of tyramine in rabbits, cats, and dogs. Burn and Tainter (1931) described a "cocaine paradox", in that cocaine potentiated the responses to adrenaline but antagonized the responses to tyramine in a wide variety of tissue preparations. Tainter (1930) showed that the typical "cocaine paradox" also applied to the metabolic effects of adrenaline. Cocaine increased the hyperglycemic responses of rabbits to adrenaline, and decreased those to ephedrine. The inhibition of tyramine by cocaine was later explained by

extensive evidence to show that the effect of tyramine was almost entirely due to the release of noradrenaline (Burn, 1932; Burn & Rand, 1958a; Bjrablaya et al., 1958), and that the action of cocaine was to inhibit this release (Burn & Rand, 1958b).

Because of the similarities of supersensitivity due to cocaine and denervation, common mechanisms have been proposed by many authors (Rosenblueth, 1932; Fleckenstein & Bass, 1953; Innes & Kosterlitz, 1954b; and Trendelenburg et al., 1962).

The subject of supersensitivity has been extensively reviewed from time to time (Cannon & Rosenblueth, 1949; Furchgott, 1955; Trendelenberg, 1963 & 1966), and various hypothesis have been proposed for its mechanism. The hypotheses proposed specifically for the mechanism of cocaine supersensitivity will be briefly discussed.

1. Enzyme Inhibition

Blaschko et al. (1937) showed that extracts of rat liver, kidney, and intestine caused the oxidation of adrenaline. They postulated that an amine oxidase present in these tissues was responsible for the metabolism of adrenaline. Philpot (1940) showed that the local anesthetics cocaine and procaine were strong inhibitors of monamine oxidase activity in guinea pig liver suspensions. Peralta & Lizaralde (1946) found that cocaine prolonged the chronotropic response to adrenaline and suggested that this was due to a delay in the destruction of adrenaline. Burn (1952) suggested that the potentiation caused by cocaine was due to inhibition of monamine oxidase. Since then a considerable body of evidence has accumu-

lated to discount this hypothesis as the cause of supersensitivity to catecholamines. Brown & Hey (1956) found that cocaine was only a weak inhibitor of monamine oxidase in liver slices and defibrinated cat blood. In addition several more potent inhibitors of monamine oxidase than cocaine fail to potentiate adrenaline or noradrenaline. Iproniazid, a potent inhibitor of monamine oxidase, did not potentiate the actions of adrenaline on the nictitating membrane (Griesemer et al., 1953). Alpha cocaine did not modify the actions of adrenaline or noradrenaline on the blood pressure or nictitating membrane of the spinal cat even though it was as effective as cocaine in inhibiting monamine oxidase (Foster et al., 1955). In another study Varagic (1956) observed that isopropylisoniazide, a potent monamine oxidase inhibitor, did not modify the effect of hypogastric nerve stimulation while cocaine caused potentiation.

Furchgott (1955) suggested that another enzyme was responsible for reducing the meta-hydroxyl group of adrenaline and noradrenaline; this enzyme could be competitively inhibited by cocaine and could thus account for the cocaine potentiation. Axelrod (1957) isolated an enzyme from rat liver which catalyzed the O-methylation of adrenaline and noradrenaline. He also demonstrated the presence of the methoxy derivatives metanephrine and normetanephrine in the urine. He concluded that an enzyme, catechol-O-methyl transferase, (COMT) transfers a methyl group from S-adenosylmethionine to one of the phenolic groups of adrenaline or noradrenaline. Bacq et al., (1959) showed that in rat liver homogenates

catechol inhibited the inactivation of adrenaline by COMT. They postulated that the inhibition is responsible for the sensitization of smooth muscle to adrenaline by various ortho, di, or tri phenols. However, the blockade of COMT by cocaine was ruled out as an explanation for cocaine supersensitivity by the following studies. Crout (1961) showed that the inhibition of COMT by pyrogallol did not potentiate, but moderately prolonged the cardiovascular responses to noradrenaline in dogs. The prolonged increases in blood pressure and contractile force were associated with delayed disappearance of injected noradrenaline from the circulation. Wylie et al. (1960) showed by spectrofluorometric measurement of the accumulation of the metabolite metanephrine, that cocaine was inactive as an inhibitor of COMT. In a more recent study Missala (1966) showed that in rats the urinary metabolites of $^{14}C$-adrenaline were not altered by cocaine.

It has been shown then, that inhibition of neither of the main metabolic pathways can account for potentiation by cocaine.

2. Removal of Inhibition

Fleckenstein & Bass (1953) developed a hypothesis to explain cocaine and denervation supersensitivity. They assumed that the continuous release of transmitter substance from the nerve terminals in the nictitating membrane places the effector cells in a state of accommodation or subsensitivity. They suggested that chronic denervation or blockade by cocaine abolished this release and therefore reduced accommodation and increased sensitivity.

However, they did not take into account the earlier observations made by Rosenblueth & Rioch (1933), which showed that cocaine did not abolish release, but potentiated the response of the nictitating membrane to sympathetic nerve stimulation. Strong evidence against this hypothesis has also been presented by several other authors. Furchgott (1955) exposed aortic strips to small concentrations of adrenaline and noradrenaline which were sufficient to maintain a small contraction for one half hour. The addition of cocaine to the bath caused a large rapid contraction which could not be due to a loss of accommodation of the smooth muscle. Cervoni & Kirpekar (1966) showed that infusions of adrenaline or noradrenaline did not affect the responses of the decentralized nictitating membrane to subsequent exposures of noradrenaline and adrenaline. It has also been shown that cocaine does not impair the release of noradrenaline on nerve stimulation (Trendelenberg, 1959).

Interest in the idea of loss of inhibition as an explanation for cocaine supersensitivity has been renewed by Barnett et al. (1968). They showed that cocaine increased the maximum response of the isolated vas deferens to noradrenaline, and postulated that the potentiation was due to prevention of a desensitizing process of auto-inhibition.

3. Uptake Hypothesis

The uptake hypothesis attributes supersensitivity to an increased catecholamine concentration at the receptor as a result

of blockade of neuronal uptake which normally limits the concentration near the receptor.

Trendelenberg (1959) showed that cocaine increased the response of the nictitating membrane of the spinal cat to both preganglionic nerve stimulation and to injections of noradrenaline. Cocaine also increased both the blood pressure response to noradrenaline and the plasma concentration of noradrenaline. On the basis of this evidence he suggested that cocaine potentiates noradrenaline by delaying its inactivation, by limiting either the cell permeability to noradrenaline or its binding of noradrenaline. Macmillan (1959) proposed a hypothesis to explain the mechanism of cocaine supersensitivity based on evidence from experiments on isolated atria, perfused rabbit ear, and the heart lung preparation. He suggested that the disappearance of noradrenaline from the blood is in part due to its uptake into a store in the heart and blood vessel wall. Cocaine would potentiate noradrenaline by preventing this uptake, thus making more noradrenaline available to act on the sympathetic receptor. He also suggested, as had Burn & Rand (1958b), that cocaine blocks the effect of tyramine by preventing the release of noradrenaline.

Furchgott et al. (1963) proposed a unitary hypothesis to explain the "cocaine paradox". The actions and interactions of noradrenaline, tyramine and cocaine were studied in isolated rabbit aorta and guinea pig atria. Cocaine potentiated the effects of noradrenaline and antagonized the effects of tyramine. Tissues pretreated with reserpine and which were consequently depleted of noradrenaline

did not respond to tyramine, but in these tissues large concentrations of tyramine were as effective as cocaine in potentiating the responses to noradrenaline. Exposure of reserpinized tissues to noradrenaline restored the responses to tyramine. However, this restoration was prevented by the presence of either tyramine or cocaine in the bath. On the basis of these findings Furchgott postulated a specific transport mechanism located in the nerve cell membrane which moves noradrenaline into the cell where it is stored or metabolized. The findings were explained by cocaine combining with this transfer site and thus competitively blocking the uptake of both noradrenaline and tyramine. This would increase the concentration of noradrenaline at the receptor causing a potentiated response and would prevent tyramine from entering the cell to release noradrenaline. Similarly tyramine could combine with the transfer site, block uptake of noradrenaline, thus increase noradrenaline concentration and potentiate the response.

The uptake hypothesis is supported by evidence that noradrenaline is taken up into the sympathetic nerve endings and this uptake is blocked by cocaine. Whitby et al. (1961) found that the uptake of $^{3}$H-noradrenaline was greatest in tissues with a rich sympathetic innervation. It was selectively taken up by the heart, spleen, and adrenals of the cat. The same workers showed that the tissue uptake of $^{3}$H-noradrenaline after denervation of the superior cervical ganglion was severely reduced (Hertting et al. 1961a).

Direct evidence for the localization of noradrenaline uptake in sympathetic nerves was shown by fluorescence microscopy

(Malmfors, 1965). He showed that noradrenaline was taken up by adrenergic nerve fibres in the iris of reserpine-treated rats. Gillespie & Kirpekar (1966) studied the localization of noradrenaline in the cat spleen, using both fluorescence microscopy and autoradiography; noradrenaline was located in the nerve fibres among the smooth muscle of the capsule, trabeculae, arteries and veins.

Direct evidence for inhibition of noradrenaline uptake by cocaine has been provided by a number of workers. Whitby et al. (1960) and Hertting et al. (1961b) showed that cocaine markedly reduced the uptake of circulating $^{3}$H-noradrenaline into the heart and spleen of the cat, and increased the plasma levels. Iversen (1967) stated that cocaine caused 50% inhibition of the uptake of $^{3}$H-noradrenaline in the isolated perfused rat heart. Lindmar & Muscholl (1964) showed that the isolated perfused rabbit heart removed 40% of the noradrenaline in the perfusate, and cocaine reduced this to 10%. In a similar study Gillespie & Kirpekar (1965) found that 29% of the noradrenaline infused into splenic arterial blood of the cat was recovered in the venous blood. After treatment with cocaine the recovery was increased to 81%. The blockade of noradrenaline uptake has also been shown histochemically using fluorescence microscopy in the rat iris (Hillarp & Malmfors, 1964; Malmfors, 1965) and in the central artery of the rabbit ear (De la Lande et al., 1967).

Further evidence in support of the uptake theory of cocaine supersensitivity was obtained from experiments on sympathetic

nerve stimulation. Cocaine has been shown to potentiate the responses of the nictitating membrane of the cat to nerve stimulation (Rosenbleuth & Rioch, 1933; Trendelenberg, 1959; Innes & Kosterlitz, 1954a; Kirpekar & Cervoni, 1963; Haefely et al., 1964). In addition to the potentiation of nerve stimulation, cocaine should promote the overflow of transmitter on stimulation of sympathetic nerves if its potentiating effect is due to the blockade of neuronal uptake. However, the results of various studies have been conflicting. Trendelenberg (1959) and Blakely et al. (1963) found that cocaine did not affect the liberation of noradrenaline from splenic nerves after stimulation. Kirpekar & Cervoni (1963) found that in the cat spleen cocaine caused only a slight increase in the output of noradrenaline after low frequency stimulation and did not change the output after high frequency stimulation. However, the increase at low frequencies was confirmed in experiments by Thoenen et al. (1964) and Haefely et al. (1965), where cocaine did increase the venous outflow of noradrenaline from the cat spleen stimulated at low frequencies.

Many attempts have been made to correlate the blockade of uptake and the degree of supersensitivity. Trendelenberg (1959) showed that the pressor response to noradrenaline in the spinal cat was related to the plasma noradrenaline concentration. Cocaine increased both the pressor response to noradrenaline and the plasma concentration of noradrenaline. Muscholl (1961) found an inverse linear correlation between the noradrenaline content of the rat heart and the blood pressure response to noradrenaline.

In another study the pressor response to noradrenaline in the pithed rat was potentiated more by cocaine than the pressor response to adrenaline. Concomitantly cocaine caused a larger decrease in the level of $^{3}$H-noradrenaline than $^{3}$H-adrenaline in the heart and spleen (van Zwieten,1965). Trendelenberg (1966) found that cocaine reduced the uptake of $^{3}$H-noradrenaline in the cat nictitating membrane by 66%. The same cocaine concentration was previously shown to cause a 23-fold increase in the sensitivity of the nictitating membrane to noradrenaline (Trendelenberg, 1965).

Stafford (1963) found that cocaine increased the rate and force of contraction of isolated guinea pig atria caused by noradrenaline and adrenaline but not isoproterenol. She suggested that cocaine does not potentiate isoproterenol because isoproterenol has no affinity for the storage sites. Bhagat et al. (1967) showed that cocaine inhibited the uptake of $^{3}$H-noradrenaline in isolated guinea pig atria and increased the chronotropic response to noradrenaline. The chronotropic response to isoproterenol, however was not potentiated by cocaine. Anden et al. (1964) showed spectrofluorometrically that isoproterenol, in contrast to noradrenaline and adrenaline, was not taken up by the isolated rabbit heart. In the same study isoproterenol failed to restore the sympathomimetic effects of tyramine and was not potentiated by cocaine. In contrast, Callingham & Burgen (1966) showed that $^{3}$H-isoproterenol is taken up by the isolated perfused rat heart. Both the total uptake and retention of isoproterenol were small compared to noradrenaline. However, cocaine did not

block the uptake of isoproterenol. The same type of results were found in the dog heart by Hardman et al. (1965). Cocaine markedly potentiated the actions of noradrenaline and adrenaline on the contractile force and phosphorylase activity in the dog heart in situ, while the response to isoproterenol was not potentiated. In the same study cocaine decreased the amount of $^{3}$H-noradrenaline and $^{3}$H-adrenaline found in the heart at the peak of the contractile force, but had no effect on the amount of $^{3}$H-isoproterenol in the heart.

The observation that cocaine does not potentiate the effects of noradrenaline and adrenaline in denervated structures is taken by many workers as evidence in support of the uptake hypothesis. Hertting et al. (1961a) showed that the uptake of noradrenaline was severely impaired by chronic denervation. Cocaine does not potentiate the response to noradrenaline in the chronically denervated nictitating membrane (Kukovetz & Lembeck, 1961; Haefely et al., 1964a), or in chronically denervated rabbit atria (Furchgott et al., 1963). These authors attribute the loss of cocaine potentiation to the absence of an uptake mechanism for noradrenaline after denervation. Innes & Kosterlitz (1954b) also showed that cocaine failed to potentiate adrenaline or noradrenaline in chronically denervated nictitating membranes, but attributed this finding to both cocaine and degeneration of the postganglionic fibres causing the same alterations in the effector system.

Iversen (1967) stated that the uptake process for catecholamines in rat heart showed structural and stereochemical specificity.

Noradrenaline was taken up more rapidly than adrenaline and the l isomers of both amines accumulated more rapidly than the d isomers. Trendelenberg (1965), and Tye et al. (1967) found that the sensitization of the nictitating membrane of the cat by cocaine had the same structural and stereospecificity found by Iversen. They concluded that these results support a causal relationship between supersensitivity and blockade of uptake since cocaine sensitizes the nictitating membrane more to those amines which are taken up readily than those which are not. However, in a more recent study (Trendelenberg et al.,1968) showed that there was no stereospecificity for the uptake of the d and l isomers of noradrenaline in the isolated perfused rabbit heart; cocaine blocked the uptake of these isomers equally well. However, in isolated rabbit atria cocaine potentiated the positive inotropic effect of l noradrenaline but not d noradrenaline. These results negate the earlier findings and show that the sensitizing effect of cocaine is not related to selective blockade of uptake of either isomer of noradrenaline.

4. Inconsistencies of the Uptake Hypothesis

The blockade of neuronal uptake has become accepted by most workers as the mechanism for cocaine supersensitivity. However, there are a number of observations which are inconsistent with the uptake hypothesis.

Maxwell et al. (1966) found that the potentiating effect of cocaine was not proportional to its ability to block the uptake of noradrenaline. Cocaine caused a 30% reduction in the uptake of

noradrenaline in isolated rabbit aorta before any potentiation was observed. When uptake was reduced from 30-70% the response to noradrenaline increased as a function of the percentage reduction in uptake. However, cocaine caused considerable increases in the response to noradrenaline without any further reduction in uptake.

Marks et al. (1967) found that the time required for saturation of the uptake of $^{3}$H-cocaine in isolated vas deferens was much greater than the time required to reach maximum potentiation of the response to noradrenaline. On the other hand the disappearance of $^{3}$H-cocaine after washing the tissue was much faster than the disappearance of the cocaine potentiation.

Bevan & Verity (1967) showed that cocaine potentiated noradrenaline in a nerve free preparation. The adventitial layer was removed from the rabbit aortic strip. Histological observations showed the strips to be devoid of innervation. The strips were also considered denervated as they did not respond to tyramine or transmural stimulation. They concluded that cocaine is able to potentiate the responses to noradrenaline in the absence of neuronal uptake. In a similar study Kalsner (1966) showed that cocaine potentiated responses to noradrenaline in rabbit aorta which had been stored at $4^{\circ}$C for 10 days. The aortic strips did not respond to tyramine and were therefore considered denervated. However, the absence of neuronal uptake was not demonstrated in either of these studies.

Kalsner (1966) also showed that cocaine potentiated the response to methoxamine in the isolated rabbit aortic strip, while

Iversen (1967) demonstrated that methoxamine is not taken up by the nerve endings in the rat heart.

Kalsner (1966) and Innes (unpublished) have shown that cocaine potentiates the responses to histamine in rabbit aorta and spleen respectively, while Day & Stockridge (1964) showed that cocaine did not inhibit the uptake of histamine by mast cells.

Isaac and Goth (1965) showed that the antihistamine pheninda-mine effectively blocked the uptake of noradrenaline in the rat heart, but did not enhance the chronotropic responses to noradrena-line in isolated atria.

Davidson & Innes (1968) showed that cocaine potentiated the contractile response to isoproterenol in the cat spleen but did not alter the neuronal uptake of isoproterenol.

Because of these inconsistencies it seems impossible to correlate the blockade of uptake and cocaine supersensitivity. While blockade of uptake may play a role in the potentiating action of cocaine it is not the only mechanism operating.

5. Receptor or Postreceptor Action of Cocaine

Clark (1937) suggested that cocaine potentiates adrenaline by altering the receptor in some manner so that either the rate of its association with adrenaline is increased or its rate of dissociation is decreased. Maxwell et al. (1959) showed that in the dog, cocaine prevented the competitive blockade of the pressor response to noradrenaline by phentolamine, but did not effect the non-equilibrium blockade by Dibenamine. From these results and a

study previously described (Maxwell et al., 1966), they postulated that cocaine potentiates noradrenaline by acting directly on the receptors in the smooth muscle cells. Lewis & Miller (1966) showed that cocaine did not protect against $^{3}$H-phenoxybenzamine blockade of the response to noradrenaline in the rat seminal vesicle. The amount of radioactivity in the tissues exposed to cocaine and $^{3}$H-phenoxybenzamine was not different from tissues exposed to $^{3}$H-phenoxybenzamine alone.

Karr (1966) showed that in isolated spleen strips exposed to cocaine the simultaneous presence of large concentrations of phentolamine completely prevented supersensitivity to noradrenaline from developing. Similarly large concentrations of noradrenaline reduced the degree of potentiation of noradrenaline by cocaine. Tyramine, which has a greater affinity than phentolamine for the neuronal uptake site, failed to alter the cocaine potentiation. Therefore the blockade of uptake could not account for the effect of cocaine. However, both phentolamine and noradrenaline have high affinities for the adrenergic receptor and either prevented or reduced the cocaine potentiation. It was therefore suggested that cocaine induced an allosteric change in the adrenergic receptor which increased the receptor affinity for noradrenaline, resulting in a potentiated response. Green & Fleming (1967) studied the affinities of noradrenaline and two alpha receptor antagonists by determining the $pA_2$ values for noradrenaline-phentolamine antagonism, and the $pD_2$ values for noradrenaline-phenoxybenzamine antagonism in the cat nictitating membrane. Supersensitivity pro-

duced by cocaine did not alter either the $pA_2$ or the $pD_2$ values. The authors suggested that cocaine therefore does not affect the adrenergic receptor, and supersensitivity is due to blockade of neuronal uptake. However, if cocaine altered the affinities of the receptor for the agonist and antagonist to an equal extent, no changes in either $pA_2$ or $pD_2$ would have been seen if the adrenergic receptor were affected by cocaine.

Rosenblueth (1932) showed that cocaine potentiated the responses to acetylcholine in the cat nictitating membrane. This was later confirmed by Koppanyi & Feeney (1959) who postulated that cocaine potentiates acetylcholine by causing changes in the muscle cell permeability to ions.

6. Possible Role of Calcium in Supersensitivity

Bevan & Verity (1967) suggested that the potentiating effect of cocaine was due to its direct action on the vascular smooth muscle cell where it caused the labilization of calcium loosely bound to the cell membrane. Such an effect of cocaine has also been suggested by Daniel & Wolowyk (1966). They showed that very large concentrations of cocaine caused contraction of the isolated rabbit uterus. These responses were not due to an action mediated by alpha receptors or muscarinic receptors since they were not blocked by phenoxybenzamine, tolazoline, or atropine. Depolarization by potassium, Na-EDTA or Mg-Na-EDTA, or immersion in a calcium-free solution, did abolish the contraction induced by cocaine. Strontium chloride restored the response to cocaine in

a calcium free solution.

Calcium has also been implicated in supersensitivity induced by denervation and reserpine. Gutmann & Sandow (1965) found that caffeine-induced contracture in rat skeletal muscle was potentiated by chronic denervation. They suggested that denervation alters the intracellular binding of calcium, making it more easily released by caffeine.

Carrier & Shibata (1967) showed that the aorta obtained from young rabbits, 5-6 weeks old, were severely depleted of calcium by pretreatment with reserpine, and showed supersensitivity to noradrenaline. However, the aorta from older rabbits, 12 weeks old, were not depleted of calcium and did not show any supersensitivity to noradrenaline. It was suggested that reserpine either increases the availability of ionized calcium or increases the excitability and permeability of the membrane. Hudgins & Fleming (1966) found that the supersensitivity due to reserpine in isolated rabbit aorta was unspecific. Reserpine not only increased the response of the tissue to noradrenaline but also to acetylcholine and potassium. Phentolamine in a concentration which provided marked antagonism of the responses to noradrenaline had little or no effect on the responses to acetylcholine or potassium. It was postulated that reserpine changes the responsiveness of the smooth muscle cell at some point beyond the receptor. Further support for this hypothesis was obtained in a more recent study by Westfall & Fleming (1968), who found the chronotropic responses to noradrenaline or calcium in the dog heart lung preparation were increased by reserpine pretreatment. The response to noradrenaline was

antagonized by propranolol but the response to calcium was not. They suggest that the postreceptor change induced by reserpine could in some way involve calcium.

## B. THE ROLE OF CALCIUM IN SMOOTH MUSCLE CONTRACTION

### 1. General Evidence

The initial evidence which indicated that calcium was necessary for muscular contraction was obtained from studies on cardiac muscle. Ringer (1883) demonstrated that the frog heart failed to contract and remained relaxed when calcium ions were omitted from the perfusion fluid. Locke and Rosenheim (1907) showed that contraction and the utilization of glucose by isolated rabbit hearts were dependent on calcium. Mines (1913) was able to dissociate the mechanical and electrical responses in isolated frog hearts. He observed that the heart maintained its electrical activity when made mechanically inactive by the lack of calcium.

Sandow (1952) was the first to postulate a definite role for calcium in excitation-contraction coupling of skeletal muscle. He proposed that depolarization of the cell membrane triggers the release of calcium which then causes activation of the contractile elements. He further postulated that the activation of the contractile elements was due to the enzymatic activation of a myosin-ATPase system by calcium. This hypothesis is supported by Niedergerke (1955) and Caldwell & Walster (1963) who showed that the intracellular application of calcium in heart and skeletal muscle caused shortening of the muscle fibres.

An increased influx of $^{45}Ca$ has been shown to occur during a potassium-induced contracture of frog sartorius muscle (Bianchi & Shanes, 1959), and frog rectus abdominis muscle (Shanes, 1961).

These authors postulated that this increased entry of calcium underlies the development of the contractile response.

Frank (1964 a,b, & 1965) showed that contraction of skeletal muscle involved in addition to the utilization of extracellular calcium, the release of calcium from a binding site within the muscle.

The role of calcium in the contraction of skeletal muscle has been extensively reviewed (Sandow, 1965; Podolsky, 1965; Peachy, 1968; Caldwell, 1968). However, a brief description of the theories proposed for the mechanism by which calcium is involved in activation of the contractile elements (Davies, 1963)and relaxation (Webber, 1963) in skeletal muscle is warranted since the mechanism is not as well delineated in smooth muscle. These theories propose that membrane depolarization by ions, electrical stimulation, or drugs is transmitted in some way to the sarcoplasmic reticulum. This electrical disturbance then triggers the release of calcium ions from the reticulum. The released calcium then interacts with the actomyosin system and initiates ATP splitting and contraction. Relaxation results from the removal of calcium from the actomyosin, an active process involving the splitting of ATP, then causes calcium to be reaccumulated into the sarcoplasmic reticulum. It is also possible that the reaccumulation of calcium in the sarcoplasmic reticulum is brought about by binding of calcium by a muscle relaxing factor. The structure of skeletal muscle and the relationship of chemical and physical factors involved in contraction are fairly well established;

Huxley's sliding filament scheme for contraction has been generally accepted.

The exact role of calcium in the activation of smooth muscle is far from clear, and the mechanism of contraction of smooth muscle is not fully understood. However, the mechanism of contraction of smooth muscle may be similar to that of skeletal muscle. Actomyosin has been demonstrated in vascular smooth muscle (Filo et al., 1963) and in uterus (Needham & Williams, 1963). However, smooth muscle contains much less actomyosin than does skeletal muscle. Smooth muscle has been described by Needham & Shoenberg (1964) and Needham (1964) as consisting of cells which are tightly packed with filaments lying parallel to the long axis of the cell; these filaments have approximately the same diameter as the actin filaments in skeletal muscle. However, myosin filaments have not been clearly discerned. Relaxed and contracted taenia coli have been compared by electron microscopy. Cross sectional examination showed that the diameter of the filaments in contracted cells was no greater than the diameter of the filaments in relaxed cells. However, the total number of filaments was much greater in contracted than relaxed cells. If the filaments slide closer together, an increase in their number would be seen in a cross section. It is therefore, presumed that some sort of sliding filament mechanism exists in smooth muscle, which is similar to the mechanism generally accepted to account for skeletal muscle contraction. The occurence of sarcoplasmic reticulum in smooth muscle was established by electron microscopic examination of the rabbit uterus (Shoenberg,

1958). However, there was no relationship seen between the sarcoplasmic reticulum and the myofilaments or the cell membrane, as has been shown for skeletal muscle.

## 2. Electrical Activity

It is well established that calcium affects the electrical activity of smooth muscle. Bülbring & Kuriyama (1963a) measured spontaneous electrical activity in guinea pig taenia coli with microelectrodes. They found that lowering the calcium concentration of the bathing solution decreased the resting membrane potential, and the amplitude and rates of rise and fall of the action potential. In a similar study, Bülbring & Kuriyama (1963b) found that acetylcholine depolarized the membrane and accelerated spike discharge. These effects did not occur in the absence of calcium and were enhanced by the presence of excess calcium. Adrenaline, which relaxes taenia coli, abolished spike activity without hyperpolarizing the membrane. This inhibitory effect was potentiated by excess calcium and abolished in the absence of calcium. Marshall (1965) reported that in rat uterus a decrease in extracellular calcium caused membrane depolarization, and reduced spike height and frequency. However, an increase in extracellular calcium increased the membrane potential and the height and rate of rise of the action potential.

## 3. Dissociation of Electrical and Mechanical Activity

There is a great deal of evidence that electrical changes

in the smooth muscle membrane i.e. depolarization, are not required for contraction to occur. Various smooth muscles, already depolarized by potassium, have been shown to respond to drug stimulation. Rabbit and guinea pig ileum, rat uterus, and chick amnion contracted in response to acetylcholine (Robertson, 1960); guinea pig taenia coli contracted in response to carbachol (Durbin & Jenkinson, 1961); rat uterus contracted in response to acetylcholine (Edman & Schild, 1961 and 1962); rat seminal vesicle contracted in response to adrenaline, and rabbit uterus contracted in response to acetylcholine (Edman & Schild, 1963).

Sue et al. (1964) simultaneously recorded isometric tension and membrane potentials from isolated rabbit pulmonary arteries. They found that sympathetic nerve stimulation or administration of noradrenaline caused contraction of the artery in the absence of action potentials, and without any change in the membrane potential. Keating (1964) used a sucrose gap technique to measure membrane potentials of spiral strips of sheep carotid artery. He found that noradrenaline, adrenaline, and histamine all depolarized the membrane and caused contraction of the muscle. However, arteries depolarized by potassium contracted in response to these drugs without producing any electrical changes.

In more recent studies Axelsson et al. (1967) and Johansson et al. (1967) found calcium to be essential for both activation of the contractile mechanism and maintenance of electrical activity of isolated rat portal vein. Exposure of the muscle to potassium chloride caused depolarization and a sustained contraction.

Noradrenaline further increased this contraction without causing any changes in membrane potential. When the muscle was placed in a calcium-free solution both mechanical and electrical activity were abolished. Noradrenaline re-established both electrical and mechanical activity, but the magnitude of the responses was much less than seen in solutions with normal calcium content. However, when the calcium chelating agent EGTA was added to the calcium-free solution noradrenaline failed to restore electrical or mechanical activity. These results were interpreted as showing that the main effects of noradrenaline on tension in portal vein are mediated through changes in the pattern of electrical activity. They suggested that noradrenaline, however, could influence the contractile mechanism in a way independent of propagated or non-propagated potential changes. They further suggested that noradrenaline restored the electrical and mechanical activity in a calcium-free solution by liberating loosely or tightly bound calcium. These various pools of calcium will be discussed later in the introduction.

4. Extracellular Calcium

It is well established that the contractile response in smooth muscle is lost or reduced in the absence of calcium, and within limits the magnitude of the response is a function of the extracellular calcium concentration.

Bozler (1960) showed that extracellular calcium was required for smooth muscle contraction. Spontaneous contractions of isolated

frog stomach were abolished when the preparation was bathed in an isomotic sucrose solution with no calcium present. The contractions were re-established after the addition of calcium chloride.

Urakawa & Holland (1964) reported that contractures caused by potassium in isolated taenia coli were accompanied by enhanced uptake of $^{45}Ca$. However, contrary results were obtained by Schatzman (1964) in taenia coli, and Van Breemen & Daniel (1966) in rat uterus. They found that neither potassium nor acetylcholine altered the uptake of $^{45}Ca$, but did cause a net efflux of $^{45}Ca$. In other studies Bauer et al. (1965) showed that the uptake of $^{45}Ca$ was increased in taenia coli by applying additional tension to the muscle; and Goodford (1965) found that depolarization of taenia coli by potassium did not alter the efflux of $^{45}Ca$. The discrepancies between these various studies are difficult to explain, but they may be due to variations in technique.

Contractions of rabbit aortic strips by adrenaline (Briggs & Melvin, 1961) and by potassium sulphate (Briggs, 1962) were accompanied by a net influx of $^{45}Ca$. However, adrenaline did not affect tension or cause the uptake of $^{45}Ca$ in glycerol extracted muscle. In another study Briggs & Hannah (1965) reported that magnesium and not calcium was necessary for the contraction induced in glycerinated uterine muscle by ATP. It is assumed that only the contractile elements are present in glycerol extracted smooth muscle. They also showed the presence of a magnesium-activated ATPase which was not stimulated by calcium. However EDTA, which chelates calcium more strongly than magnesium, and EGTA which specifically chelates

calcium, prevented the contraction of the glycerinated smooth muscle. Contraction was restored by the addition of calcium. These results indicate that calcium must be available for contraction to take place, but its exact role in contraction is not clear.

Waugh (1962, 1965) found that the contractile response to adrenaline or potassium was prevented by the addition of Na- or Mg-EDTA to the perfusate of an isolated dog intestinal artery. The addition of Ca-EDTA restored responses to both noradrenaline and potassium. The contraction of the artery in response to calcium chloride was potentiated by prior noradrenaline or potassium contractions. These results were interpreted to mean that extracellular calcium is essential for excitation-contraction coupling, and that noradrenaline or potassium increase the membrane permeability to extracellular calcium.

The contraction of depolarized smooth muscle by drugs has also been shown to depend on extracellular calcium. Robertson (1960) showed that rabbit and guinea pig ileum, rat uterus, and chick amnion depolarized by potassium contracted in response to acetylcholine. The removal of calcium from the bathing solution reversibly abolished the acetylcholine response. The re-establishment of the acetylcholine contraction by the addition of calcium was prevented by EDTA. It is suggested that the acetylcholine contraction was due to an increased membrane permeability to calcium. Similar studies by Durbin & Jenkinson (1961), and Edman & Schild (1961) also showed that depolarized smooth muscle is dependent on extracellular calcium for contraction. Schild (1964)

suggested that contractions induced by drugs in smooth muscles depolarized by potassium may be due to the release of a bound calcium store in addition to the utilization of extracellular calcium.

Divalent cations such as strontium and barium have been shown to substitute for calcium in the contraction of smooth muscle.

Daniel (1963) found that either strontium or barium restored acetylcholine contractions in calcium-depleted polarized or depolarized rat uterus. The effects of these two ions lasted only as long as they were present in the bath, but the effects of restoration of calcium to the bathing fluid persisted for a long time after the calcium was subsequently removed from the bath. However, either strontium or barium alone caused contraction of the uterus bathed in a normal calcium solution. It has also been reported that contractions due to barium in taenia coli are calcium dependent (Karaki et al., 1967), and are not due to the release of noradrenaline as they were not blocked by adrenergic blocking agents (Shibata et al., 1968).

Bohr (1964) showed that the response to noradrenaline in rabbit aortic strips was greatly reduced in the absence of calcium. Barium was more potent than calcium in re-establishing the response to adrenaline, and strontium much less effective than calcium, while magnesium was ineffective. Hinke (1965) obtained different results in the rat tail artery; barium was able to substitute for calcium in a potassium-induced contraction but not in a noradrenaline-induced contraction.

Sperelakis (1962) showed that maximal tensions were developed in response to electrical stimulation of polarized and potassium-depolarized cat intestine. Higher voltage strengths were needed in the depolarized muscle. The responses of both the normal and depolarized muscle were dependent on the presence of calcium. Strontium not only substituted for calcium in the contraction of the depolarized muscle, but allowed stimulation to produce a larger contraction than in the presence of calcium. However, strontium did not substitute for calcium in the response of the polarized muscle. Sperelakis suggested that calcium has three sites of action: the cell membrane where it lowers membrane excitability; coupling of excitation and contraction; and a direct effect on the contractile elements. The observations could be accounted for by strontium substituting for calcium in the contractile elements, but not in the membrane or coupling processes.

Frank (1962) showed that strontium and other divalent cations restored potassium-induced contractures of skeletal muscle when they were added to a calcium free solution. He indicated that these divalent cations did not participate directly in contraction, but restored excitation-contraction coupling by releasing bound cellular calcium. In another study on skeletal muscle Caldwell & Walster (1963) injected various multivalent ions directly into a single muscle fibre of the leg muscle of the crab. They found that both strontium and barium caused contractions. They are in agreement with Frank, and suggest that the action of these ions is through the displacement of bound calcium from the sarcoplasmic

reticulum.

Daniel (1965) suggested that the restoration of drug-induced contractions by strontium or barium in smooth muscle is due to their substitution for calcium which is loosely bound to the outside of the membrane (superficial), and which then penetrates the membrane on depolarization. Once the ions pass through the cell membrane they displace calcium from a tightly bound store (sequestering), which then interacts with the contractile elements.

5. Bound Calcium

A number of different workers have proposed that the contractile elements of smooth muscle can be activated by calcium released from a bound intracellular site. Kolodny & Van der Kloot (1961) suggested that in the absence of extracellular calcium contraction is brought about by the release of calcium from a bound store. They found that after long periods of bathing in a calcium-free sucrose solution the frog stomach contracted in response to acetylcholine, adrenaline, and electrical stimulation, and the guinea pig taenia coli contracted in response to acetylcholine and electrical stimulation.

Imai & Takeda (1967) found that the mechanical response of taenia coli to potassium sulphate consisted of two distinct phases, an initial rapid rise of tension, and an ensuing sustained contracture. When the tissue was bathed in a calcium-free solution the rapid phase was abolished, but the sustained phase persisted, though considerably diminished in size. The addition of multi-

valent cations such as nickel, cadmium, cobalt, or magnesium to the bath augmented the sustained phase but did not restore the initial rapid phase. When potassium contractures were induced in a normal calcium solution, these cations abolished the rapid phase and depressed the sustained phase of contraction due to inhibition of calcium influx during depolarization. It was therefore suggested that the rapid initial phase and part of the sustained phase depend upon extracellular calcium, while most of the sustained phase was due to the release of bound calcium.

Hurwitz et al. (1967a) reported that longitudinal fibres from guinea pig ileum contracted when they were transferred from a physiological solution containing calcium to a calcium-free solution. The magnitude and duration of the contractile response was enhanced if the muscle was preincubated in a high calcium (36mM) solution. The addition of acetylcholine or potassium caused a further contraction of the muscle. When the tissue was incubated with $^{45}Ca$ and then transferred to a calcium-free solution, the contraction was accompanied by an increased efflux of $^{45}Ca$. It was suggested that acetylcholine, and high potassium and a calcium-free solution increase smooth muscle tone by mobilizing calcium from an intracellular compartment which may reside in the fibre membrane. Similar conclusions were reached by Van Breemen & Daniel (1966). They found that both acetylcholine and potassium increased $^{45}Ca$ efflux in rat uterus. They concluded that myometrial contraction is induced by a release of calcium from the inside of the cell membrane or the endoplasmic reticulum,

or both.

6. Multiple Calcium Stores

Recently evidence has been presented which indicates that both extracellular and bound calcium are utilized by the contractile elements for contraction of smooth muscle.

Hurwitz et al. (1967b) found that both extracellular and cellular calcium are mobilized to activate the contractile elements in smooth muscle. Isolated guinea pig ileum contracted when the bathing solution was changed from one with a high (36mM) calcium concentration; to a calcium-free solution. When the period of incubation in the high calcium solution was lengthened, the muscles accumulated increasingly more calcium, presumably in an intracellular site, and exhibited increasingly larger contractile responses when placed in a calcium-free solution. The contractile response was enhanced when acetylcholine was added to the calcium-free solution. When acetylcholine was added to a bathing solution containing a high calcium concentration a sustained contraction was obtained. Comparisons were then made between acetylcholine contractions obtained in the presence of intracellular calcium and in the presence of extracellular calcium. In one case the tissue was incubated in a high calcium solution, then bathed in a calcium-free solution containing acetylcholine; the contraction which developed was therefore dependent on an intracellular calcium store. In the second case the tissue was first depleted of calcium by being bathed in a calcium-free solution containing EDTA, then stimulated

with acetylcholine in a calcium-containing solution; the contraction which developed was therefore dependent on extracellular calcium. This contraction was immediately abolished when the calcium was removed from the bathing solution.

The muscle with a high level of intracellular calcium maintained tone for a longer period of time in the absence of extracellular calcium than did the muscle which was calcium-depleted. It was suggested that the extracellular calcium serves as a reservoir of calcium which supports muscle contraction by maintaining the cellular store in a state of partial or full saturation. It was speculated that extracellular calcium ions reach the cytoplasm of the muscle by more than one pathway. Part of the calcium might enter the cytoplasm by way of the intracellular store, and a second part might enter by an alternate route which bypasses the intracellular depot. However, these pathways are not necessary for an explanation of the results. The main point is that two different calcium stores can be utilized for the contraction of smooth muscle.

Hinke (1965) showed that potassium and noradrenaline contract vascular smooth muscle by utilizing different stores of calcium. The contraction of isolated perfused rat tail artery was measured as a reduction in flow at a constant perfusion pressure. A sustained contraction was obtained when either potassium or noradrenaline were added to the perfusate. When calcium was omitted from the perfusate the potassium-induced contraction decayed slowly, but the noradrenaline-induced contraction was sustained. The

potassium-induced contraction was rapidly abolished when the artery was perfused with a calcium-free solution containing EDTA, but the artery still contracted in response to noradrenaline, although with reduced magnitude. Prolonged perfusion of the artery with a calcium-free solution containing EDTA did, however, abolish the noradrenaline contraction. The contraction was rapidly restored after the addition of calcium to the perfusate. Addition of calcium to the perfusate in increments gradually restored both noradrenaline and potassium contractions which had been abolished by calcium-free plus EDTA perfusion.

Hinke suggested that vascular smooth muscle contains at least two calcium fractions, both of which may be bound to the cell surface. One calcium fraction is loosely bound and easily mobilized and is dependent on extracellular calcium. The other calcium fraction is tightly bound, and is independent of extracellular calcium. Therefore potassium-induced contractions utilize the loosely bound calcium, while noradrenaline induced contractions utilize both tightly bound and loosely bound calcium.

Daniel (1965) proposed that there are two, and possibly three, binding sites for calcium in smooth muscle. These sites are: a superficially (loosely bound) site located on the outside of the cell membrane; a sequestering (tightly bound) site located on the inside of the cell membrane; and a third site located inside the cell which might constitute microcrystalline deposits, or mitochondria. He proposed various models to explain the relationships which might exist between these binding sites. First, it is

possible that the two sites, superficial and sequestering, are in series with each other. The second possibility is a parallel arrangement where extracellular calcium reaches the sequestering sites by a pathway independent of the superficial binding sites. In addition calcium may be able to reach the cytoplasm by diffusion down its electrochemical gradient through pores which are not available until calcium is removed from the superficial binding sites. The third possibility is that both the series and parallel models are operative. A fourth possibility takes into consideration the third binding site which is located intracellularly and is in series with the sequestering sites.

## 7. Conclusion

There is no direct evidence for the existence of multiple calcium stores in smooth muscle or for the way in which drugs affect these stores. However, the studies discussed here provide strong circumstantial evidence for the existence of at least three stores of calcium in smooth muscle; extracellular calcium, calcium loosely bound to the external surface of the cell membrane, and calcium tightly bound to the internal surface of the cell membrane. This evidence is based on the fact that the calcium chelators EDTA and EGTA do not penetrate the cell membrane and bind only calcium which is extracellularly located.

Bianchi (1965) found that EDTA did not penetrate the cells of frog rectus abdominis and sartorius muscles. The $^{14}$C-EDTA space was found to be the same as the $^{14}$C-sucrose space. He

also found that $^{14}$C-EDTA washed out of the muscles at a much faster rate than did $^{14}$C-sucrose.

Van Breemen et al. (1966) showed that either EDTA or EGTA caused an increased efflux of $^{45}$Ca from rat uterus. However, they suggest that this was not due to chelation of extracellular calcium since bathing the uterus in a calcium-free solution caused a reduction in $^{45}$Ca efflux. They suggest that EDTA reversibly damages the cell membrane and thus increases the permeability to calcium. However, the increased efflux could be due to an increased membrane permeability as the result of the removal of loosely bound calcium from the membrane, and the subsequent release of calcium from an intracellular binding site as was suggested by Bianchi (1965) for skeletal muscle.

EDTA has also been shown to cause contraction of isolated uterus. These contractions were not mediated by noradrenaline or acetylcholine since they were not affected by adrenergic or cholinergic blocking agents (Daniel & Irwin, 1965). He suggested that these contractions were due to the removal of calcium from the surface of the cell membrane which in some way caused the liberation of calcium from an intracellular binding site.

The effects seen with these chelating agents are due to calcium chelation and not the chelation of magnesium. EDTA has been shown to bind calcium much more strongly than magnesium and EGTA specifically binds calcium (Chaberek & Martell, 1959). In another study Bozler (1955) showed that in glycerol extracted skeletal muscle EDTA preferentially bound calcium, and only bound magnesium after all

the calcium had been removed from the muscle.

## C. STATEMENT OF THE PROBLEM

The uptake hypothesis has become generally accepted as the mechanism of cocaine potentiation of catecholamines, despite the inconsistencies discussed above and the circumstantial nature of the evidence for a cause and effect relationship. This study was done to see if there was an alternative explanation for the mechanism of cocaine supersensitivity.

It has been suggested by a number of authors discussed above that cocaine causes supersensitivity by an action on the receptor or on a postreceptor event. The activation of the smooth muscle receptor by a drug initiates a chain of events which leads to contraction. One of the links in this chain is calcium. Calcium is essential for the contraction of smooth muscle and its utilization by the contractile elements takes place after the receptor has been activated. The potentiating effects of cocaine, therefore, might be due to an alteration in the utilization of calcium for contraction so that calcium becomes more readily available to the contractile elements.

Experiments were designed primarily to see if cocaine altered the utilization of calcium for contraction of the isolated spleen strip by noradrenaline and histamine. However, any alterations in the calcium utilization could also be attributed to an increased noradrenaline concentration at the receptor as a result of release of endogenous catecholamines or the blockade of neuronal uptake. Therefore experiments were also done in order to eliminate these effects as contributing to a change in calcium utilization caused by cocaine.

# METHODS

1. Preparation of the Isolated Spleen Strip

Cats (0.3 - 3 kg) of either sex were killed by a blow on the head. The spleen was immediately removed and immersed in Krebs-Henseleit solution at 4°C. The spleen was placed on an inverted Petri dish covered by a filter paper saturated with Krebs-Henseleit solution. Strips 20 mm long and 2 mm wide were cut from the peripheral edge of the spleen after removal of adhering fat tissue. Each strip was suspended in a muscle bath containing 15 ml of bathing solution kept at 37.5°C, and bubbled with a gas mixture of 95% $O_2$ and 5% $CO_2$. Isotonic contractions against 1 g tension were recorded on a kymograph at ten times lever magnification at a paper speed of 1 mm/min. The strips were allowed to equilibrate in the bath for one hour before drugs were added. The bathing fluid was replaced every fifteen minutes except when drugs were present in the bath.

2. Bathing Solutions

The following bathing solutions were made with distilled demineralized water.

i. Standard Krebs-Henseleit solution: NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $KH_2PO_4$ 1.1, $MgSO_4$ 1.2, $NaHCO_3$ 25.0, and glucose 11.0 mM.

ii. In experiments where the tissues were to be bathed in a medium without calcium, a "Ca-Free" solution was prepared by omission of $CaCl_2$ from the standard Krebs-Henseleit formula. In order to promote the depletion of tissue calcium the chelating agent disodium ethylenediaminetetra-

acetic acid (EDTA) was added to the Ca-Free solution in a concentration of 0.3 mM, and designated a "O-Ca EDTA" solution.

3. Drugs

Stock solutions of all drugs were made in concentrations of 1, 5, or 10 mg/ml and stored at 4°C. l-Noradrenaline bitartrate, and tyramine hydrochloride were dissolved in 0.01N HCl. Cocaine hydrochloride, procaine hydrochloride, desmethylimipramine, histamine acid phosphate, ethylenediaminetetra-acetic acid (EDTA) disodium and dicalcium salts, strontium chloride, and calcium chloride were dissolved in distilled demineralized water.

A stock solution of reserpine in a concentration of 5 mg/ml was made; 100 mg reserpine was dissolved in 2.0 ml glacial acetic acid, 2.5 ml propylene glycol, and 2.5 ml 95% ethanol and sufficient water added to make 20 ml.

Diazoxide was dissolved in distilled demineralized water made alkaline with 0.1 N NaOH to facilitate solution.

All drugs were diluted to appropriate concentrations on the day of use with 0.9% sodium chloride, in the case of catecholamines 0.01N HCl was added to prevent rapid oxidation.

Drugs were added to the muscle bath with 0.01 to 0.5 ml micropipettes. Drug concentrations are expressed as final concentration in the muscle bath in g/ml except calcium and strontium, which are expressed in molar concentrations. The weights of noradrenaline, histamine, and tyramine are expressed in terms of the free base, all other drugs as weight of the salt.

4. Treatment with Reserpine

Animals were treated with 1.0 mg/kg intraperitoneally twenty-four hours before an experiment. Spleen strips were shown to be depleted of noradrenaline either by spectrofluorometric assay or by the loss of response to tyramine $3 \times 10^{-5}$ g/ml.

5. Assay for Catecholamines

Noradrenaline and adrenaline were assayed fluorometrically by a modification of the methods described by Bertler et al. (1958) and Euler and Lishajko (1959). The catecholamines were purified, concentrated, and then transformed into fluorescent compounds by oxidation and rearrangement in alkali to the trihydroxyindoles, adrenolutine and noradrenalutine.

Spleen strips weighing 100-200 mg were removed from the bath and placed in test-tubes containing 10 ml of cold ($4^{o}C$) Krebs-Henseleit solution. After 10 to 30 minutes the tissues were blotted with filter paper, weighed, and immediately homogenized with 5 ml of 0.4 N perchloric acid per gram of tissue. The homogenates were then made up to 10 ml with 0.4 N perchloric acid and centrifuged at 8500 RPM at $0^{o}C$ for 10 minutes.

Na-EDTA 0.1 g was added to the supernatant and the pH adjusted to 6.2 to 6.3 with 5 N potassium carbonate. The homogenates were further purified with a Dowex 50S-X8 cation exchange resin (500 mg, 20 $mm^2$ x 12 mm). Before use, the column was washed through with 20 ml 2 N HCl, 20 ml of sodium acetate buffer pH 6.0 containing Na-EDTA, and again with 20 ml of deionized water. The extracts were then filtered into the column; the column was washed with 20

ml of deionized water and eluted with 10 ml 1N HCl. The flow rate through the column was kept constant at 0.25 ml/min.

5 ml of the eluate was brought to pH 6.2 to 6.3 with 5 N potassium carbonate. The noradrenaline and adrenaline in the eluate were then oxidized to their chrome derivatives by adding 0.1 ml of 0.25% potassium ferricyanide for three minutes. The oxidation was then interrupted and the chrome derivatives converted into fluorescent lutines by the addition of 2 ml of a mixture of 9 ml to 5 N NaOH, 1 ml 2% ascorbic acid and 0.2 ml diaminoethane.

The extracts were then immediately read on a Farrant optical spectrofluorometer. Noradrenaline and adrenaline are differentiated by making use of their different activation and fluorescent spectra. The activation peaks for adrenolutine and noradrenolutine are 435 and 395 Mu respectively, the corresponding fluorescence peaks being 540 and 490 Mu respectively. Tissue blanks were treated in the same way as the samples except that no potassium ferricyanide was added. Standards of adrenaline and noradrenaline as well as a reagent blank were run in parallel with the samples. To control the possible presence of interfering material a known amount of adrenaline and noradrenaline were added each to one portion of the eluate and treated in the same way as the sample.

The amounts of adrenaline and noradrenaline were calculated with the aid of the following formula (Cohen & Goldenberg, 1957). The assay was sensitive to 0.002 $\mu$g/g of tissue.

$$Y = \frac{(m\ A_b/A_a) - n}{N_a\ A_b/A_a - N_b}$$

$$X = \frac{n - YN_b}{A_b}$$

Where:

$Y$ = Noradrenaline concentration in μg

$X$ = Adrenaline concentration in μg

$m$ = Fluorescent value for adrenaline (tissue sample-blank)

$n$ = Fluorescent value for noradrenaline (tissue sample-blank)

$A_a$ = Adrenaline standard - blank measured at the adrenaline wave lengths

$A_b$ = Adrenaline standard - blank, measured at the noradrenaline wave lengths

$N_a$ = Noradrenaline standard-blank, measured at the adrenaline wave lengths

$N_b$ = Noradrenaline standard-blank, measured at the noradrenaline wave lengths

## 6. Measurements and Statistics

Contraction and relaxation of the spleen strips were measured to the nearest millimetre from the kymograph record. The contractions were measured at the peak of the response after they had reached a steady state. Relaxation was calculated as the difference between the magnitude of the peak contraction in response to an agonist and the contraction remaining after 30 mm of exposure to an antagonist.

When comparisons were made between spleen strips from the same cat, or within the same spleen strip the results were analyzed by the *t*-test for paired observations (Goldstein 1964). Observations from different spleens were compared by "Student's *t*-test".

# RESULTS

## A. THE EFFECT OF COCAINE ON THE UTILIZATION OF MEMBRANE AND INTRACELLULAR BOUND CALCIUM BY NORADRENALINE AND HISTAMINE

Calcium is required for contraction of smooth muscle and its utilization in excitation contraction coupling is a postreceptor event. Noradrenaline is capable of utilizing loosely and tightly bound calcium stores for contraction (Hinke, 1965; Hudgins & Weiss, 1968) whereas histamine utilizes only loosely bound calcium stores (Hudgins & Weiss, 1968). Experiments were done to see if the potentiation of noradrenaline by cocaine involved changes in this utilization.

### 1. The Potentiation of Noradrenaline by Cocaine in the Isolated Spleen Strip Bathed in a Calcium-Free Solution

In these and subsequent experiments a Ca-Free solution was used to eliminate an extracellular source of calcium, and a O-Ca EDTA solution was used to promote the removal of interstitial or loosely bound calcium.

Cocaine ($10^{-5}$ g/ml) potentiated the response to noradrenaline ($10^{-6}$ g/ml) in a Ca-Free solution. Spleen strips were first equilibrated in a O-Ca EDTA solution for 60 minutes. Tissue calcium was then reduced by repeated additions of noradrenaline to the bath until the contraction obtained was small. The tissues were then bathed in a Ca-Free solution for 30 minutes to remove the EDTA. Noradrenaline was again added to the bath; a contraction was obtained; the bath was washed and the tissue was allowed to relax. The strip was then either treated with cocaine for 5 minutes after which noradrenaline was added to the bath in the presence of cocaine (Fig. 1-A), or

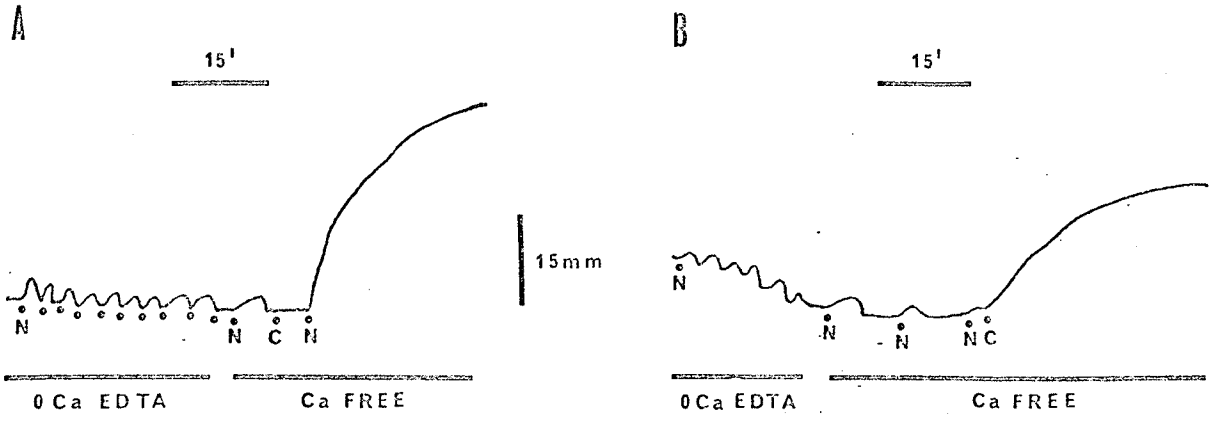


Fig. 1 The Potentiation of Noradrenaline by Cocaine in the Isolated Spleen Strip Bathed in a Calcium-Free Solution.

Two strips from the same spleen. Tissue calcium was reduced with repeated exposure to noradrenaline (N followed by dots) in a 0-Ca + EDTA solution. A. One strip exposed to cocaine (C), $10^{-5}$ g/ml, 5 min before the exposure to noradrenaline (N), $10^{-6}$ g/ml. B. One strip exposed to cocaine (C), $10^{-5}$ g/ml, at the peak of the response to noradrenaline (N), $10^{-6}$ g/ml.

cocaine was added after the response to noradrenaline had reached a plateau (Fig. 1-B). The mean differences in the heights of contraction are shown in Table 1, A & B.

In 36 strips from 32 cats, pretreatment with cocaine significantly increased the height of the noradrenaline contraction by 9.9 $\pm$ 1.3 mm. In another 47 strips from 31 cats the superaddition of cocaine significantly increased the height of the noradrenaline contraction by 9.1 $\pm$ 1.2 mm. Many of these preparations received further treatment which is described in subsequent experiments.

2. Potentiation of the Maximum Contraction to Noradrenaline by Cocaine in the Isolated Spleen Strip Bathed in a Calcium-Free Solution.

Four experiments were done on strips from four cats, each on two strips from the same spleen. Strips were first bathed in a O-Ca EDTA solution for two hours to remove tissue calcium. The bathing solution was then changed to a Ca-Free solution, to remove the EDTA. Cumulative doses of noradrenaline ($10^{-6}$ to $3 \times 10^{-4}$ g/ml) were added to the bath until a maximum contraction was obtained (Fig. 2). Cocaine ($10^{-5}$ g/ml) was added to the bath when there was no further contraction to noradrenaline. The mean differences in contraction height are shown in Table 1-C. The maximum height (21.8 $\pm$ 5.4 mm) of the noradrenaline contraction was obtained with concentrations of $3 \times 10^{-5}$ and $10^{-4}$ g/ml. Cocaine significantly increased the height of the maximum noradrenaline contraction by 3.5 $\pm$ 1.1 mm.

TABLE 1

POTENTIATION OF NORADRENALINE BY COCAINE ($10^{-5}$g/ml) IN ISOLATED SPLEEN STRIPS BATHED IN Ca-FREE SOLUTION AND THE EFFECT OF SUBSEQUENT ADDITION OF Na-EDTA ($10^{-3}$g/ml).

| Treatment | Dose of Noradrenaline g/ml | Change in Noradrenaline Contraction Due to Cocaine Mean $\pm$ S.E. mm | P | Change in Noradrenaline-Cocaine Contraction Due to Na-EDTA Mean $\pm$ S.E. mm | P |
|---|---|---|---|---|---|
| A | $10^{-6}$ | +9.8 $\pm$ 1.3 (36) | <0.001 | | |
| B | $10^{-6}$ | +9.1 $\pm$ 1.2 (47) | <0.001 | | |
| C | maximum | +3.5 $\pm$ 1.1 (8) | <0.02 | | |
| D | $10^{-6}$ | +6.8 $\pm$ 1.7 (8) | <0.01 | -5.4 $\pm$ 1.9 | <0.025 |
| E | $10^{-5}$ | +12.6 $\pm$ 4.0 (8) | <0.02 | -11.4 $\pm$ 2.8 | <0.01 |
| F | $10^{-6}$ | +28.1 $\pm$ 4.0 (4) | <0.01 | -11.8 $\pm$ 2.2 | <0.02 |
| G | $10^{-5}$ | +22.8 $\pm$ 2.8 (4) | <0.005 | -11.3 $\pm$ 2.0 | <0.02 |

B,C,D,E,F,G, : Cocaine Added After Noradrenaline had Induced Contraction
A : Cocaine Added 5 Min Before Noradrenaline
A,B,D,E, : Tissue Calcium Depleted with Noradrenaline and Na-EDTA ($10^{-4}$g/ml)
C,F,G, : Tissue Calcium Depleted with Na-EDTA ($10^{-4}$g/ml)
F,G, : Spleen Strips from Reserpine Treated Cats

The Numbers in Parenthesis Represent the Number of Spleen Strips.

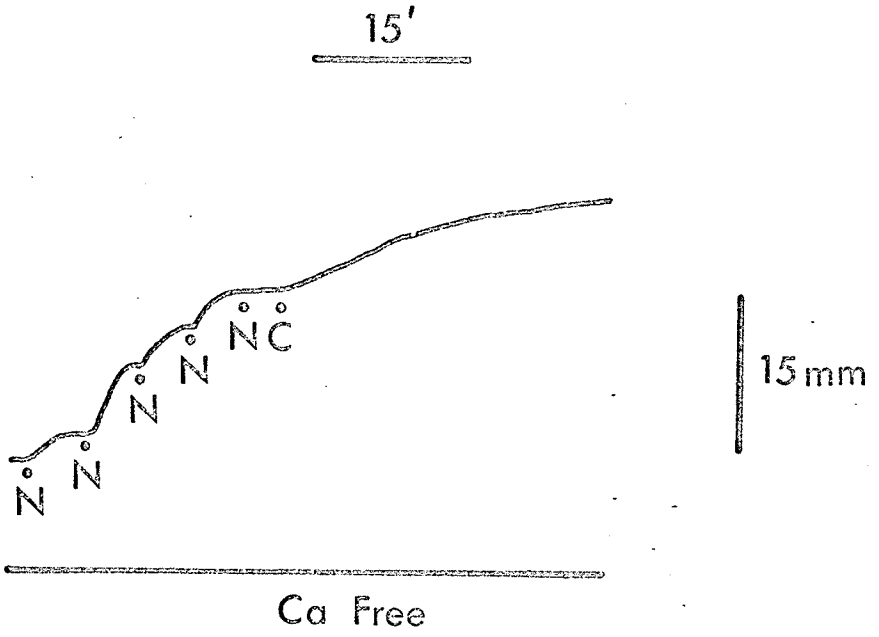


Fig. 2 Potentiation of the Maximum Contraction to Noradrenaline by Cocaine in the Isolated Spleen Strip Bathed in a Calcium-Free Solution.

Spleen strip exposed to noradrenaline (N) in cumulative concentrations of $10^{-6}$, $3 \times 10^{-6}$, $10^{-5}$, $3 \times 10^{-5}$, $10^{-4}$ g/ml, followed by cocaine (C), $10^{-5}$ g/ml.

3. The Effect of Na-EDTA on the Potentiation of Noradrenaline by Cocaine in a Calcium-Free Solution

The above results indicate that cocaine may enable noradrenaline to utilize membrane or intracellular bound calcium more effectively. Experiments were therefore done to see if Na-EDTA would alter the potentiation of noradrenaline by cocaine in a Ca-Free solution, possibly by chelating this calcium once it was released from its binding site.

Eight experiments were done on 16 spleen strips, each with two strips from the same spleen. One strip was exposed to noradrenaline ($10^{-6}$ g/ml) and the other to ($10^{-5}$ g/ml). Tissue calcium was first reduced by addition of noradrenaline to the bath containing a O-Ca EDTA solution until the contraction obtained was small. The bathing solution was then changed to Ca-Free, to remove the EDTA. A small contraction of constant height was obtained after a number of additions of noradrenaline to the bath. This small contraction to noradrenaline ($10^{-6}$ g/ml, Fig. 3-A; and $10^{-5}$ g/ml, Fig. 3-B) was potentiated by the addition of cocaine ($10^{-5}$ g/ml) to the bath. The further addition of Na-EDTA ($10^{-3}$ g/ml) caused a small transient contraction of the tissue, then relaxation, thus significantly reducing the potentiation by cocaine. The mean differences in contraction height are shown in Table 1-D & E. The potentiation by cocaine of the larger dose of noradrenaline was not significantly greater than of the smaller dose ($P > 0.1$).

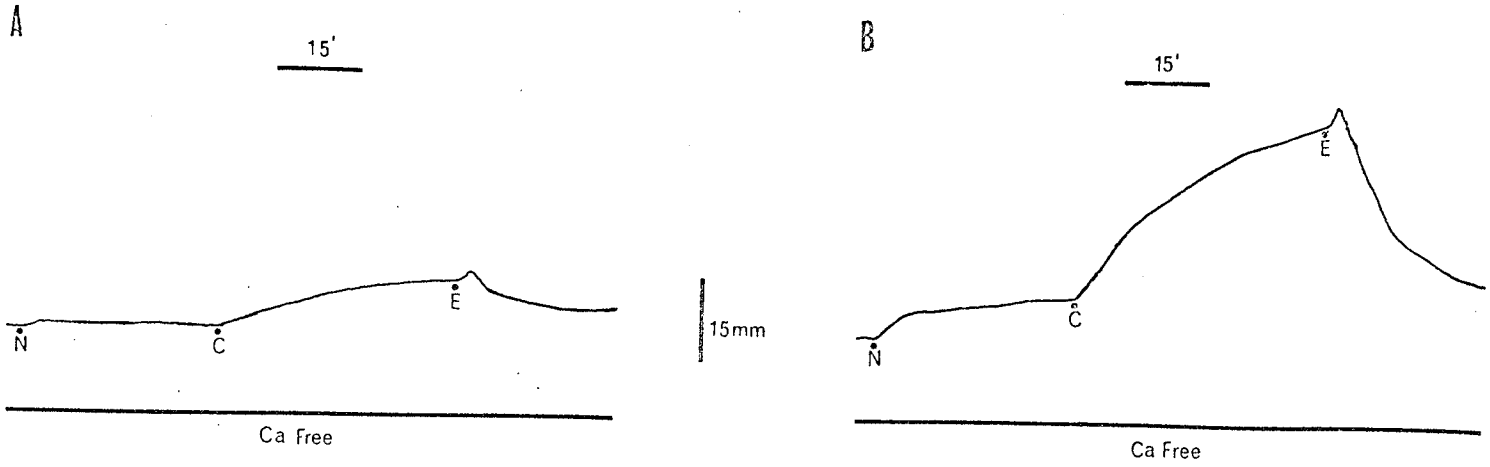


Fig. 3 The Effect of Na-EDTA on the Potentiation of Noradrenaline by Cocaine in the Isolated Spleen Strip Bathed in a Calcium-Free Solution.

A. Strip treated with noradrenaline (N), $10^{-6}$ g/ml. B. strip treated with noradrenaline (N), $10^{-5}$ g/ml. Both strips were then exposed to cocaine (C), $10^{-5}$ g/ml, followed by Na-EDTA (E), $10^{-3}$ g/ml.

4. Potentiation of Noradrenaline by Cocaine in the Reserpine-pretreated Isolated Spleen Strip Bathed in a Calcium-Free Solution

These experiments were done to show that the potentiation of noradrenaline by cocaine was not due to the release of catecholamines from nerve endings in the spleen. To deplete the spleen of noradrenaline, four cats were given reserpine, 1 mg/kg, 24 hours before the experiment. Two strips were cut from the same spleen; one strip was exposed to noradrenaline, $10^{-6}$ g/ml, (Fig. 4-A), and the other to noradrenaline, $10^{-5}$ g/ml, (Fig. 4-B). The strips were first bathed in a O-Ca EDTA solution for two hours to reduce tissue calcium. The bathing solution was then changed to Ca-Free, to remove the EDTA. Each strip was first exposed to tyramine ($3 \times 10^{-5}$ g/ml) to test for completeness of catecholamine depletion by reserpine. Both concentrations of noradrenaline caused small contractions which were greatly potentiated by the addition of cocaine ($10^{-5}$ g/ml). There was no significant difference between the degree of potentiation of the larger and smaller doses of noradrenaline by cocaine ($P > 0.3$). The further addition of Na-EDTA ($10^{-3}$ g/ml) caused the strips to relax significantly. The mean differences in contraction heights are shown in Table 1-F & G.

5. The Effect of Repeated Administration of Na-EDTA on Noradrenaline-induced Contractions of the Isolated Spleen Strip Bathed in a Calcium-Free Solution

Experiments were done on 16 spleen strips from 8 cats. Two strips were cut from the same spleen; one was exposed to noradrenaline,

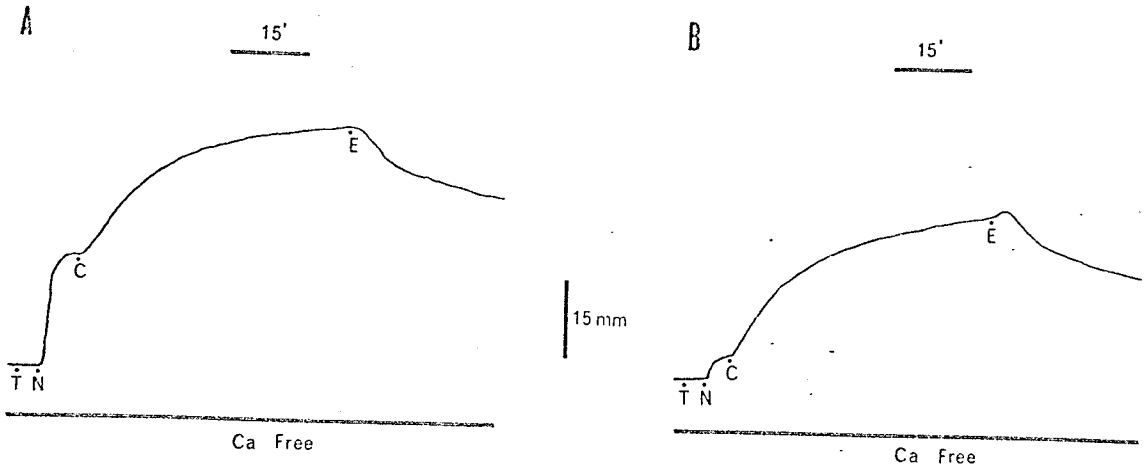


Fig. 4 Potentiation of Noradrenaline by Cocaine in the Reserpine-pretreated Isolated Spleen Strip Bathed in a Calcium-Free Solution.

Two strips from the same spleen in which reserpine, 1 mg/kg, was administered 24 hours before the experiment.
A. Strip treated with noradrenaline (N), $10^{-5}$ g/ml.
B. Strip treated with noradrenaline (N), $10^{-6}$ g/ml.
Both strips were tested with tyramine (T), $3 \times 10^{-5}$ g/ml, then exposed to noradrenaline and to cocaine (C), $10^{-5}$ g/ml, followed by Na-EDTA (E), $10^{-3}$ g/ml.

$10^{-6}$ g/ml, and the other to noradrenaline, $10^{-5}$ g/ml. Figure 5 illustrates a typical experiment. The strips were first bathed in a O-Ca EDTA solution for 2 hours to reduce the tissue calcium. The bathing solution was then changed to Ca-Free, to remove the EDTA. Exposure to noradrenaline contracted the tissue, which was relaxed by further addition of Na-EDTA ($10^{-3}$ g/ml) to the bath (Fig. 5- a & d). When the procedure was repeated 30 min later the contraction due to noradrenaline was much smaller and Na-EDTA did not cause relaxation; instead there was a small increase which was not statistically significant, (Fig. 5- b & e). The tissue was then bathed in normal Krebs-Henseleit solution to replace some of the tissue calcium. After one hour the bathing solution was changed back to Ca-Free. The same dose of noradrenaline now caused a much larger contraction than after the first exposure; these were relaxed by further addition of Na-EDTA to the bath (Fig. 5- c & f). The results were similar for both dose levels of noradrenaline. Analysis of the data is shown in Table 2.

6. <u>The Effect of Repeated Administration of Na-EDTA on the Potentiation of Noradrenaline by Cocaine in the Isolated Spleen Strip Bathed in a Calcium-Free Solution</u>

Experiments were done on 8 spleen strips from 8 cats. The strips were first bathed in a O-Ca EDTA solution for 2 hours to reduce tissue calcium. The bathing solution was then changed to Ca-Free to remove the EDTA. Noradrenaline ($10^{-6}$ g/ml) caused a small contraction which was potentiated by the addition of cocaine

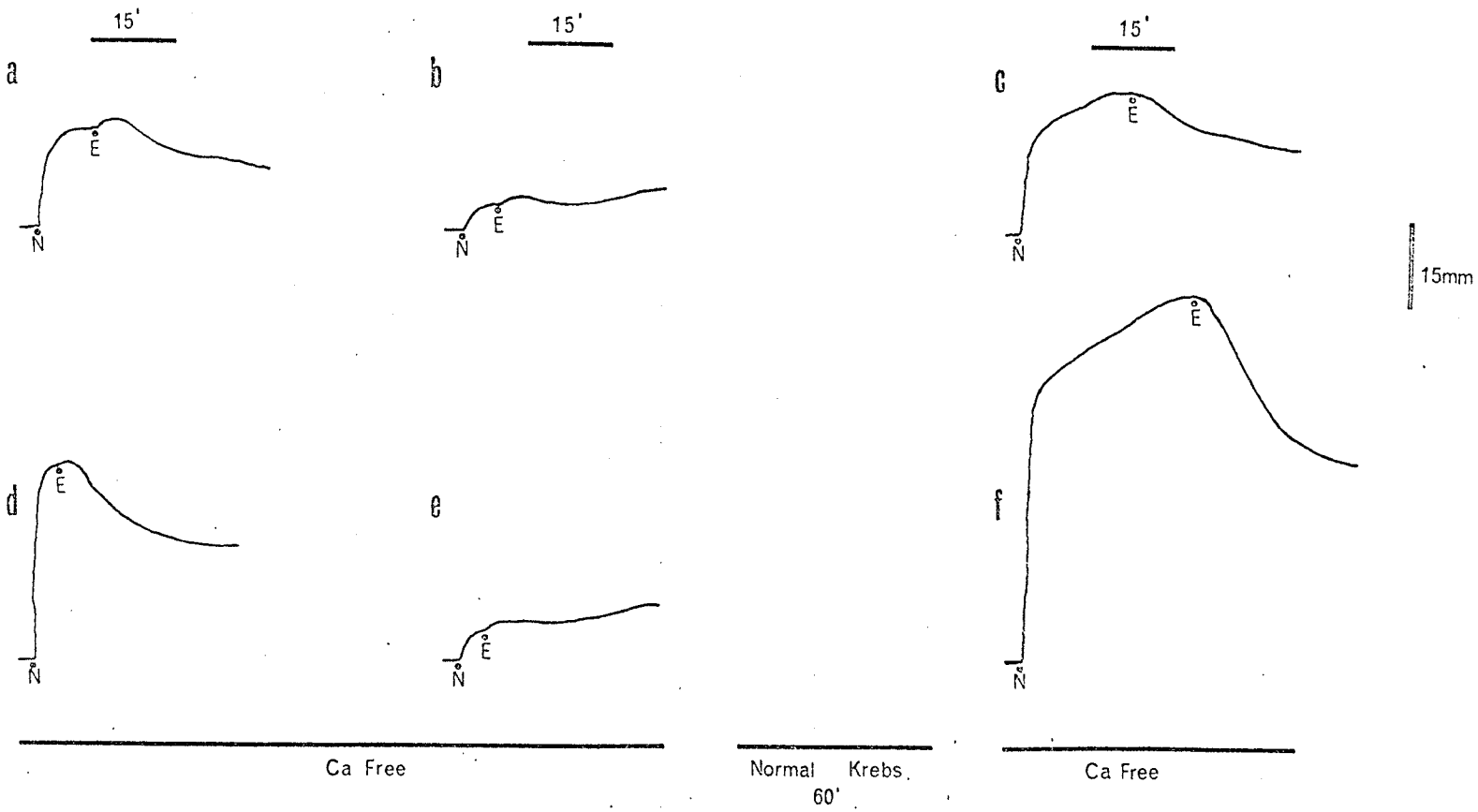


Fig. 5 The Effect of Repeated Administration of Na-EDTA on Noradrenaline-induced Contractions of the Isolated Spleen Strip Bathed in a Calcium-Free Solution.

Two strips from the same spleen. Top tracings, a, b,c, one strip treated with noradrenaline (N), $10^{-6}$ g/ml, followed by Na-EDTA (E) $10^{-3}$ g/ml. Bottom tracings, d,e,f, one strip treated with noradrenaline (N), $10^{-5}$ g/ml, followed by Na-EDTA (E) $10^{-3}$ g/ml. First exposure, a & d; second exposure, b & e; third exposure, c & f. The strips were exposed to normal Krebs-Henseleit solution for 60 min between b & c and e & f.

TABLE 2

THE EFFECT OF Na-EDTA ($10^{-3}$g/ml) ON THE NORADRENALINE-INDUCED CONTRACTION OF ISOLATED SPLEEN STRIPS BATHED IN A Ca-FREE SOLUTION.

| Treatment | Noradrenaline $10^{-6}$g/ml | | Noradrenaline $10^{-5}$g/ml | |
|---|---|---|---|---|
| | Contraction Mean ± S.E. mm | Change in Contraction Due to Na-EDTA Mean ± S.E. mm | Contraction Mean ± S.E. mm | Change in Contraction Due to Na-EDTA Mean ± S.E. mm |
| A. First Contraction | 6.6 ± 1.7 (8) | -3.3 ± 0.8 (P<0.005) | 36.6 ± 11.6 (8) | -6.0 ± 1.5 (P<0.01) |
| B. Second Contraction | 2.3 ± 0.6 (8) | +0.1 ± 0.3 (P>0.7) | 28.1 ± 11.4 (8) | +2.3 ± 1.8 (P>0.2) |
| C. Third Contraction | 9.4 ± 3.5 (8) | -4.5 ± 1.5 (P<0.02) | 56.9 ± 12.4 (8) | -19.1 ± 4.4 (P<0.005) |
| | A-B (P<0.01) | | A-B (P<0.05) | |
| | C-B (P<0.05) | | C-B (P<0.005) | |
| | C-A (P>0.02) | | C-A (P<0.001) | |

The Numbers in Parenthesis Represent the Number of Spleen Strips.

($10^{-5}$ g/ml) to the bath. The further addition of Na-EDTA ($10^{-3}$ g/ml) caused the tissue to relax, thus reducing the potentiation by cocaine (Fig. 6-a). Thirty minutes later the procedure was repeated and the noradrenaline contraction, the potentiation by cocaine, and the relaxation by Na-EDTA were all reduced (Fig. 6-b). After another 30 minutes the responses were further reduced (Fig. 6-c). The preparation was then bathed in normal Krebs-Henseleit solution to replace some of the tissue calcium. After one hour the bathing solution was changed back to Ca-Free. Exposure of the strip to the same dose of noradrenaline caused a larger contraction than before the calcium replacement and this was potentiated by the further addition of cocaine to the bath (Fig. 6-d). The addition of 2.5 mM calcium caused a further contraction. Analysis of the data is shown in Table 3.

7. The Effect of Calcium-EDTA on the Potentiation of Noradrenaline by Cocaine of the Isolated Spleen Strip

Experiments were done to see if the inhibition of the cocaine potentiation of noradrenaline by EDTA required chelation of calcium. Four experiments were done, each with two strips cut from the same spleen. One strip was tested with Na-EDTA (Fig. 7-A) and the other with Ca-EDTA (Fig. 7-B). Both strips were first bathed in a O-Ca EDTA solution for two hours to reduce the tissue calcium. The bathing solution was then changed to Ca-Free, to remove the Na-EDTA. Noradrenaline ($10^{-6}$ g/ml) caused a small contraction which was potentiated by the addition of cocaine ($10^{-5}$ g/ml) to the bath.

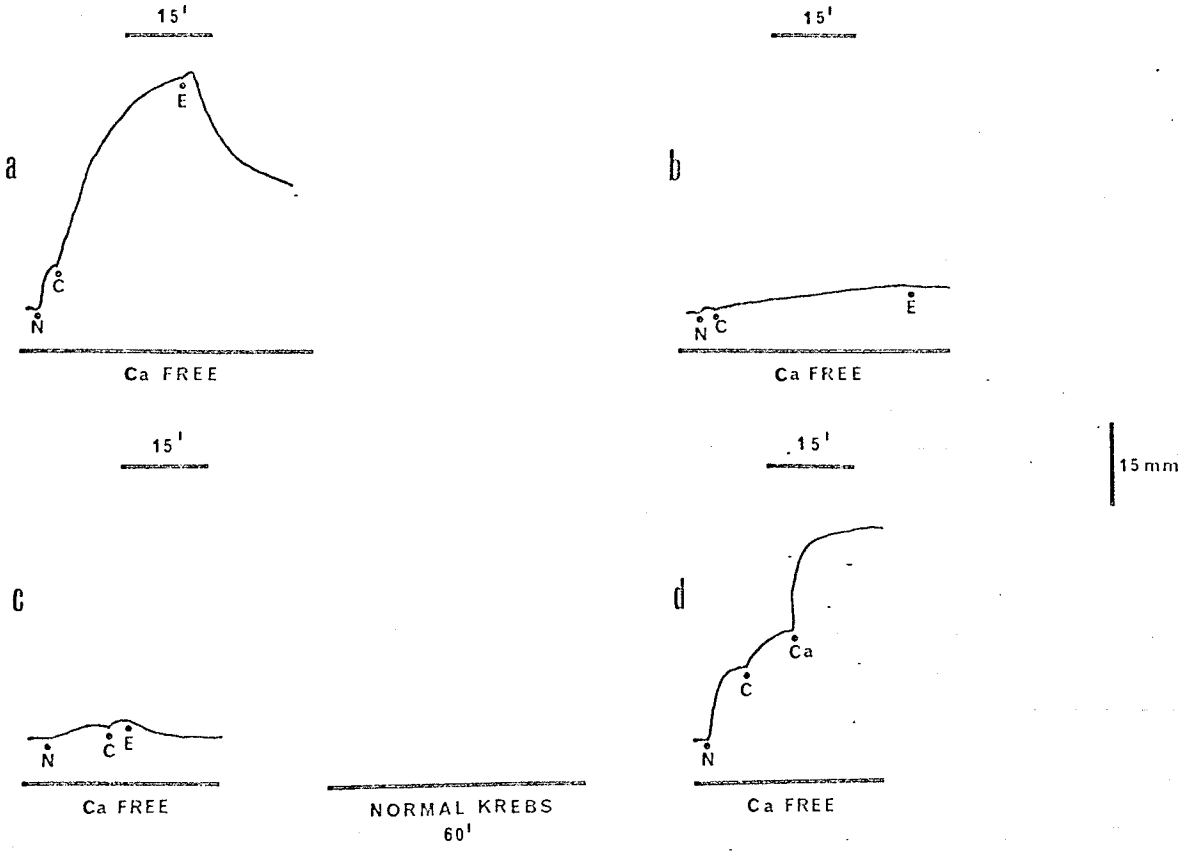


Fig. 6 The Effect of Repeated Administration of Na-EDTA on the Potentiation of Noradrenaline by Cocaine of the Isolated Spleen Strip Bathed in a Calcium-Free Solution.

One spleen strip treated with noradrenaline (N) $10^{-6}$ g/ml followed by cocaine (C), $10^{-5}$ g/ml, and Na-EDTA (E), $10^{-3}$ g/ml. a- first exposure; b- second exposure; c- third exposure; d- fourth exposure, but calcium 2.5 mM (Ca) was added instead of Na-EDTA. The tissue was exposed to normal Krebs-Henseleit solution for 60 min between c & d.

TABLE 3

THE EFFECT OF Na-EDTA ($10^{-3}$g/ml) ON THE POTENTIATION OF NORADRENALINE ($10^{-6}$g/ml) BY COCAINE ($10^{-5}$g/ml) IN ISOLATED SPLEEN STRIPS BATHED IN A Ca-FREE SOLUTION.

| Treatment | Noradrenaline Contraction Mean ± S.E. mm | Change in Contraction Due to Cocaine Mean ± S.E. mm | Change in Contraction Due to Na-EDTA Mean ± S.E. mm |
|---|---|---|---|
| A. First Contraction | 6.8 ± 1.0 (12) | +20.6 ± 4.0 ($P<0.001$) | -15.7 ± 2.8 ($P<0.001$) |
| B. Second Contraction | 2.8 ± 0.9 (12) | +2.1 ± 0.4 ($P<0.001$) | -1.1 ± 0.3 ($P<0.01$) |
| C. Third Contraction | 2.0 ± 0.7 (10) | +1.0 ± 0.1 ($P<0.001$) | -0.7 ± 0.4 ($P>0.1$) |
| D. Fourth Contraction | 13.8 ± 3.5 (8) | +7.9 ± 3.0 ($P<0.05$) | Change in Contraction Due to Ca (2.5 mM) |
| | | | +10.6 ± 2.1 ($P<0.005$) |
| | A-B $P<0.02$ | | |
| | B-C $P>0.05$ | | |
| | D-C $P<0.01$ | | |
| | D-A $P<0.02$ | | |

The Numbers in Parenthesis Represent the Number of Spleen Strips.

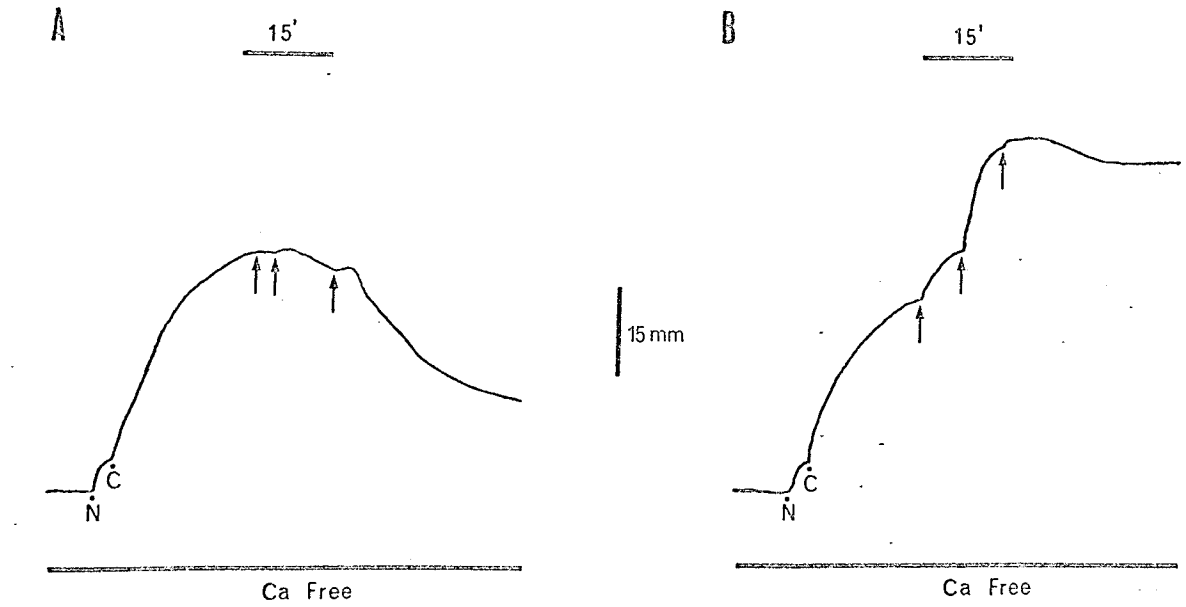


Fig. 7 The Effect of Ca-EDTA on the Potentiation of Noradrenaline by Cocaine in the Isolated Spleen Strip Bathed in a Calcium-Free Solution.

Two strips from the same spleen. A- treated with Na-EDTA. B- treated with Ca-EDTA. Both strips were exposed to noradrenaline (N), $10^{-6}$ g/ml, followed by cocaine (C), $10^{-5}$ g/ml, and then exposed to either Na- or Ca-EDTA in cumulative concentrations of $10^{-5}$, $10^{-4}$, and $10^{-3}$ g/ml at the arrows.

The tissues were then exposed to either Na-EDTA or Ca-EDTA in cumulative contractions of $10^{-5}$, $10^{-4}$, and $10^{-3}$, g/ml. The mean differences in contraction are shown in Table 4. All three concentrations of Ca-EDTA significantly increased the already potentiated contraction. The first two concentrations of Na-EDTA did not significantly change the noradrenaline cocaine contraction, but the largest concentration significantly relaxed the strip.

Four additional experiments were done with normal Krebs-Henseleit solution. There was no effect when the strips were exposed to Ca- or Na-EDTA ($10^{-3}$ g/ml). However, Na-EDTA significantly reduced the contraction to noradrenaline ($10^{-6}$ g/ml) by a mean of $3.5 \pm 0.9$ mm, $P < 0.05$. Ca-EDTA was without effect.

8. Potentiation of Histamine by Cocaine in a Calcium-Free Solution

The effect of cocaine on the histamine-induced contraction in a calcium-free solution was tested to see if the cocaine alteration of the utilization of membrane or intracellular bound calcium was specific for noradrenaline.

Experiments were done with 16 spleen strips from 8 cats. The spleen strips were first equilibrated in a O-Ca EDTA solution for one hour. Tissue calcium was then reduced by adding histamine to the bath until the contraction obtained was no more than 1 or 2 mm. The tissues were then bathed in a Ca-Free solution for 30 minutes to remove EDTA. Cocaine ($3 \times 10^{-5}$ g/ml) failed to potentiate the contraction to histamine ($10^{-6}$ g/ml) in 4 strips pretreated with cocaine for 5 minutes (Fig. 8-A), and 12 strips exposed to cocaine

TABLE 4

THE EFFECT OF Ca-EDTA ON THE POTENTIATION OF NORADRENALINE ($10^{-6}$g/ml) BY COCAINE ($10^{-5}$g/ml) IN ISOLATED SPLEEN STRIPS BATHED IN A Ca-FREE SOLUTION.

| | Ca-EDTA | | Na-EDTA | |
|---|---|---|---|---|
| Dose of EDTA g/ml | Change in Contraction Mean ± S.E. mm | P | Change in Contraction Mean ± S.E. mm | P |
| $10^{-5}$ | +6.5 ± 1.7 (4) | <0.05 | +1.8 ± 0.7 | >0.1 |
| $10^{-4}$ | +20.8 ± 3.4 (4) | <0.01 | -0.8 ± 2.2 | >0.7 |
| $10^{-3}$ | +28.0 ± 3.4 (4) | <0.005 | -20 ± 6.4 | <0.05 |

The Numbers in Parenthesis Represent the Number of Spleen Strips.

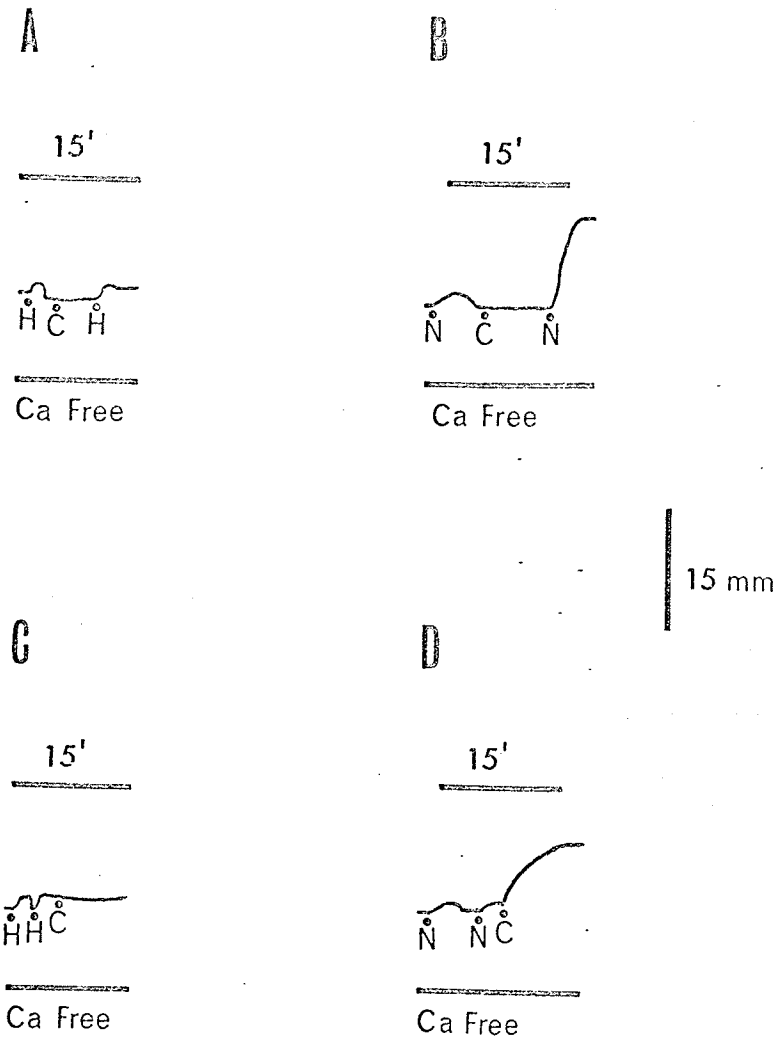


Fig. 8 The Effect of Cocaine on the Histamine-induced Contraction of the Isolated Spleen Strip Bathed in a Calcium-Free Solution.

Two spleen strips from the same spleen; top tracings from one strip and bottom tracings from the second. A- first strip treated with histamine (H), $10^{-6}$ g/ml, before and in the presence of cocaine (C), $10^{-5}$ g/ml. B- same strip treated with noradrenaline (N), $10^{-6}$ g/ml, before and in the presence of cocaine (C), $10^{-5}$ g/ml. C- second strip treated with histamine (H), $10^{-6}$ g/ml, followed by cocaine (C), $10^{-5}$ g/ml. D- same strip treated with noradrenaline (N), $10^{-6}$ g/ml, followed by cocaine (C), $10^{-5}$ g/ml.

after the histamine contraction had reached its maximum (Fig. 8-C). However, 150 minutes later cocaine was still able to potentiate the response to noradrenaline ($10^{-6}$ g/ml) in the same tissues (Fig. 8-B & D).

9. Potentiation by Noradrenaline of the Contraction to Histamine in a Calcium-Free Solution

The above results have shown that in the absence of extracellular calcium cocaine did not potentiate the response to histamine. Hudgins and Weiss (1968) reported that histamine depends mainly on extracellular calcium for contraction, whereas noradrenaline can utilize a membrane or intracellular bound calcium store. Experiments were therefore done to see if noradrenaline could make membrane or intracellular bound calcium available for histamine to utilize in contraction.

Eight experiments were done, each with two strips cut from the same spleen. One strip was exposed to cocaine ($10^{-5}$ g/ml) for 5 minutes and the other strip served as control. The strips were first bathed in a O-Ca EDTA solution for two hours to reduce tissue calcium. The bath was then changed to Ca-Free, to remove the EDTA. Small contractions of constant heights were obtained after alternate additions of noradrenaline ($10^{-6}$ g/ml) and histamine ($10^{-6}$ g/ml) to the bath. Noradrenaline then caused a small contraction in the control strip (Fig. 9-A) and a large contraction in the cocaine-treated strip (Fig. 9-B). When the responses to noradrenaline had reached their maximum the addition of histamine to the bath caused

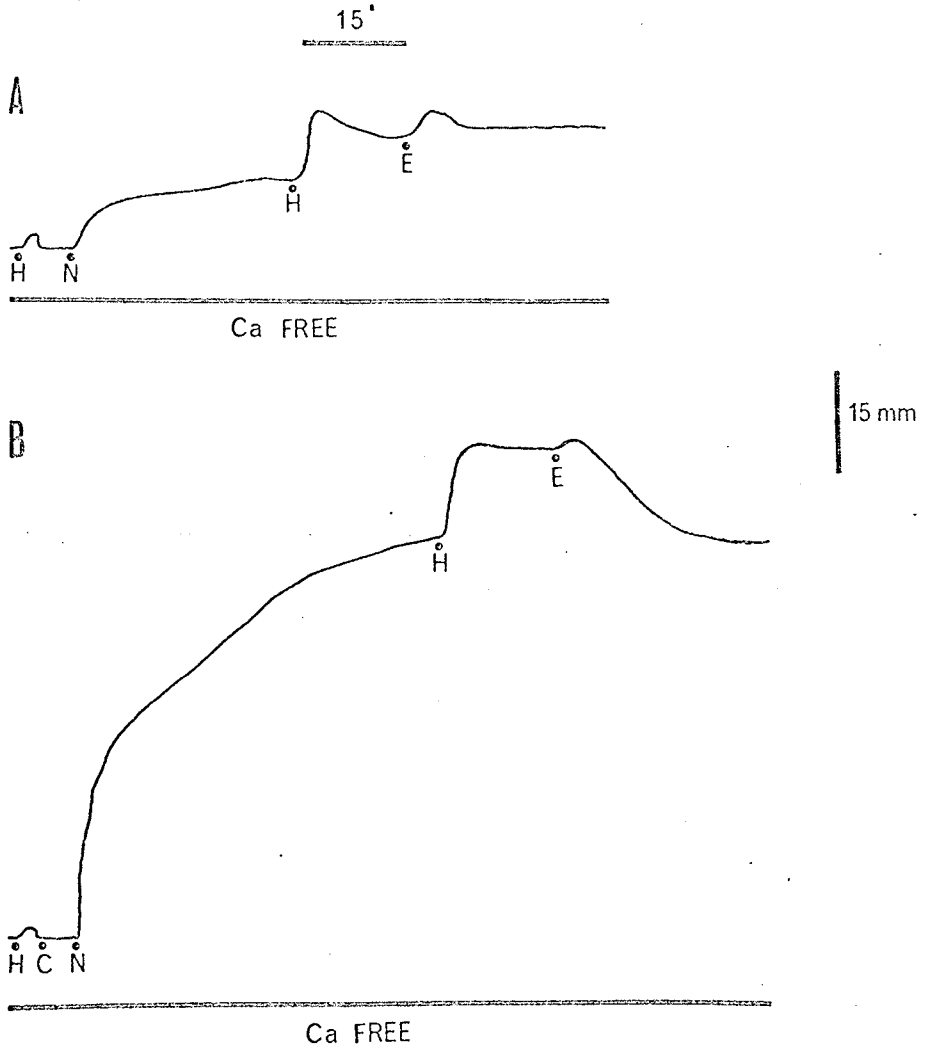


Fig. 9 Potentiation of the Histamine Contraction by Noradrenaline in the Isolated Spleen Strip Bathed in a Calcium-Free Solution.

Two strips from the same spleen. A- control strip, B- strip treated with cocaine (C), $10^{-5}$ g/ml. The strips were exposed to histamine (H), $10^{-6}$ g/ml, before and in the presence of noradrenaline (N), $10^{-6}$ g/ml, followed by exposure to Na-EDTA (E), $10^{-3}$ g/ml.

a further contraction of both strips. The addition of Na-EDTA ($10^{-3}$ g/ml) to the bath caused the control strip to contract and the cocaine treated strip to relax. The results are summarized in Table 5.

The height of the histamine contraction was significantly greater in the presence than in the absence of noradrenaline in both control and cocaine-treated strips. The difference between the histamine contractions after noradrenaline in the control and cocaine-treated strips was not significant, $P > 0.1$. Na-EDTA significantly increased the contraction in control strips and significantly reduced it in the cocaine-treated strips.

10. The Effect of Diazoxide on the Potentiation of Noradrenaline by Cocaine in Isolated Spleen Strips Bathed in a Calcium-Free Solution

Wohl et al. (1968) showed that the non-diuretic benzothiadiazine, Diazoxide, competitively antagonized restoration by calcium of the contraction caused by noradrenaline in rat aorta bathed in a calcium-free solution. This indicated that Diazoxide interfered with the utilization of calcium for contraction by noradrenaline. Experiments were therefore done to see if Diazoxide would antagonize the potentiation of noradrenaline by cocaine in a calcium-free solution.

Experiments were done in 20 spleen strips from 5 cats. Twelve strips were treated with cocaine. Tissue calcium was first reduced by adding noradrenaline, $10^{-6}$ g/ml, to the bath containing a O-Ca EDTA solution until the contraction obtained was small. The bathing

TABLE 5

POTENTIATION OF THE RESPONSE TO HISTAMINE ($10^{-6}$g/ml) BY NORADRENALINE ($10^{-6}$g/ml) IN A CALCIUM-FREE SOLUTION.

| Treatment | Change in Contraction Mean ± S.E. mm | P |
|---|---|---|
| Control | | |
| A. Noradrenaline Potentiation of Histamine Contraction | 4.25 ± 0.74 (8) | <0.001 |
| B. Na-EDTA ($10^{-3}$g/ml) Contraction | 2.43 ± 0.86 (8) | <0.05 |
| Cocaine ($10^{-5}$ g/ml) | | |
| C. Noradrenaline Potentiation of Histamine Contraction | 6.13 ± 0.77 (8) | <0.01 |
| D. Na-EDTA ($10^{-3}$g/ml) Relaxation | 6.25 ± 0.80 (8) | <0.01 |

The Numbers in Parenthesis Represent the Number of Spleen Strips.

solution was then changed to Ca-Free, to remove the EDTA. A small contraction of constant height was obtained after a number of additions of noradrenaline to the bath. Cocaine ($10^{-5}$ g/ml) was then added in 12 strips, significantly increasing the contraction (Fig. 10-a, Table 6-A). The further addition of Diazoxide ($10^{-4}$ g/ml) caused a small but significant relaxation (Table 6-B). The effect of Diazoxide without cocaine was tested in 8 strips from the same cats after similar treatment with O-Ca EDTA and Ca-Free solutions. Diazoxide ($10^{-4}$ g/ml) caused a small increase in the response to noradrenaline, but the increase was not statistically significant (Fig. 10-b, Table 6-C).

The effect of Diazoxide pretreatment on the potentiation of noradrenaline by cocaine was tested in another 8 strips from 2 cats. Tissue calcium was first reduced as described above, and the strips then bathed in a Ca-Free solution. One tissue was pretreated with Diazoxide ($10^{-4}$ g/ml) and cocaine ($10^{-5}$ g/ml) for 5 minutes and then exposed to noradrenaline ($10^{-6}$ g/ml), Fig. 10-c. The bath was then washed and the tissue allowed to relax. One half hour later the same tissue was pretreated with cocaine ($10^{-5}$ g/ml) for 5 minutes and then exposed to noradrenaline ($10^{-6}$ g/ml) Fig. 10-d. The second tissue was pretreated with Diazoxide and cocaine second, thus reversing the order of treatment.

Pretreatment with cocaine given alone significantly increased the height of the noradrenaline contraction, but pretreatment with both cocaine and Diazoxide did not cause a significant increase (Table 6-D & E). The difference between the mean increase in con-

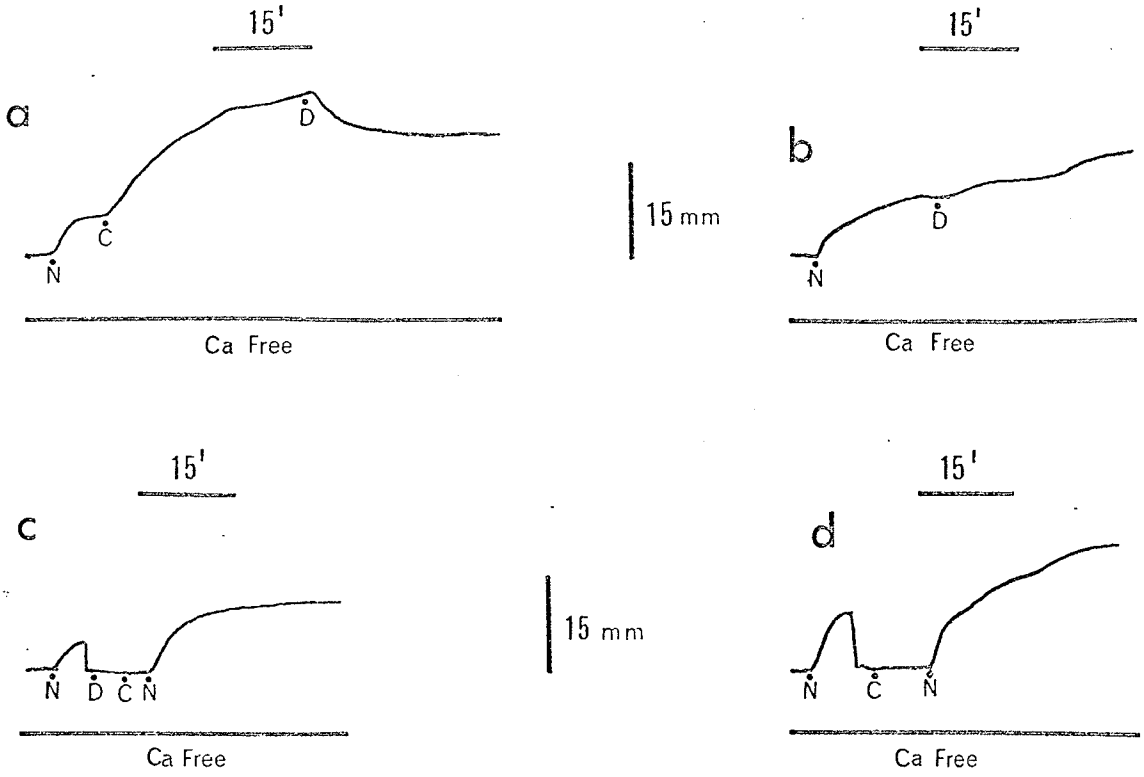


Fig. 10 The effect of Diazoxide on the Potentiation of Noradrenaline by Cocaine in the Isolated Spleen Strip Bathed in a Calcium-Free Solution.

Top tracings - two strips from the same spleen. a-strip exposed to noradrenaline (N), $10^{-6}$ g/ml, followed by cocaine (C), $10^{-5}$ g/ml, and then Diazoxide (D), $10^{-4}$ g/ml. b-strip exposed to noradrenaline (N), $10^{-6}$ g/ml.

Bottom tracings - c & d from one spleen strip. c-strip exposed to noradrenaline (N), $10^{-6}$ g/ml, before and in the presence of cocaine (C), $10^{-5}$ g/ml, and Diazoxide (D), $10^{-4}$ g/ml. d. Same spleen strip exposed to noradrenaline (N), $10^{-6}$ g/ml, in the presence of cocaine (C), $10^{-5}$ g/ml.

TABLE 6

THE EFFECT OF DIAZOXIDE ($10^{-4}$g/ml) ON THE POTENTIATION OF THE CONTRACTION TO NORADRENALINE ($10^{-6}$g/ml) BY COCAINE ($10^{-5}$g/ml) IN THE ISOLATED SPLEEN STRIP BATHED IN A Ca-FREE SOLUTION.

| | Treatment | Change in Contraction Mean ± S.E. mm | P |
|---|---|---|---|
| A | Increase in Noradrenaline Contraction due to Superaddition of Cocaine | 15.9 ± 1.3 (12) | <0.001 |
| B | Relaxation of Noradrenaline-Cocaine Contraction due to Diazoxide | 4.4 ± 0.3 (12) | <0.001 |
| C | Increase in Noradrenaline Contraction due to Diazoxide | 2.9 ± 1.4 (8) | >0.05 |
| D | Increase in Noradrenaline Contraction after Cocaine Pretreatment | 5.6 ± 1.5 (8) | <0.01 |
| E | Increase in Noradrenaline Contraction after Cocaine and Diazoxide Pretreatment | 1.8 ± 0.8 (8) | >0.05 |

The Numbers in Parenthesis Represent the Number of Spleen Strips.

traction after the two treatments was significant ($P < 0.05$). The order of treatment did not effect the results obtained. These results are very similar to the results obtained in previous sections with Na-EDTA.

11. Potentiation of the Strontium-induced Contraction by Cocaine in the Isolated Spleen Strip Bathed in a Calcium-Free Solution

Strontium has been shown to cause contraction of uterine smooth muscle (Daniel, 1963). It has also been suggested that strontium can substitute for calcium by displacing bound calcium in skeletal muscle (Frank, 1962; Caldwell & Walster, 1963), and in smooth muscle (Daniel, 1965). Experiments were therefore done to see if strontium would contract the isolated spleen strip in the absence of extracellular calcium, and whether cocaine would potentiate the response.

Experiments were done on 8 spleen strips from 4 cats given reserpine (1 mg/kg, 24 hrs previously), and 8 strips from 4 normal cats; each strip served as its own control. The reserpine treated strips were used to see if strontium caused contraction by releasing noradrenaline from storage sites in the nerve endings. Tissue calcium was reduced by exposing the normal tissues to noradrenaline ($10^{-6}$ g/ml) in the presence of a O-Ca EDTA solution, and by bathing the reserpine treated tissues in a O-Ca EDTA solution for two hours. All strips were then bathed in a Ca-Free solution. The strips from reserpine treated cats were first exposed to tyramine ($3 \times 10^{-5}$ g/ml) to test for completeness of catecholamine depletion. Four normal and 4 reserpine treated strips were exposed to cumulative concentrations of

0.16 to 12.0 mM strontium until the maximum contraction was obtained. These tissues were then re-exposed to the same strontium concentrations in the presence of cocaine ($10^{-5}$ g/ml). In 4 additional reserpine treated and 4 normal tissues from the same cats the order of treatment was reversed; the tissues were treated with cocaine first followed by the control. The results are summarized in Table 7.

The maximum contraction to strontium was not significantly different in the normal and reserpine treated strips. Cocaine significantly increased the maximum strontium contraction in both the normal and reserpine treated strips, but the effect of cocaine was significantly greater in the normal than the reserpine treated tissues. The reserpine treated strips were depleted of noradrenaline, since tyramine failed to cause a contraction. This indicates that the strontium contraction was not due to the release of noradrenaline from nerve endings in the spleen strip. Reversing the order of treatment had no effect on the results obtained.

TABLE 7

THE EFFECT OF COCAINE ON THE MAXIMUM CONTRACTION DUE TO STRONTIUM IN ISOLATED SPLEEN STRIPS BATHED IN A CALCIUM-FREE SOLUTION.

| | Normal | Reserpine | |
|---|---|---|---|
| Treatment | Maximum Contraction Mean ± S.E. mm | Maximum Contraction Mean ± S.E. mm | P |
| A. Without Cocaine | 9.13 ± 3.29 (8) | 7.50 ± 1.51 (8) | >0.6 |
| B. Cocaine ($10^{-5}$g/ml) | 29.00 ± 4.66 (8) | 15.50 ± 1.95 (8) | <0.02 |
| Mean Difference B-A | 20.13 ± 3.22 | 8.00 ± 2.28 | <0.01 |
| P | <0.001 | <0.01 | |

The Numbers in Parenthesis Represent the Number of Spleen Strips.

## B. THE EFFECT OF COCAINE ON THE UTILIZATION OF EXTRACELLULAR CALCIUM FOR CONTRACTION BY NORADRENALINE AND HISTAMINE

Waugh (1965) and Hinke (1965) have shown that noradrenaline utilizes both extracellular and membrane bound calcium for contraction of vascular smooth muscle. Briggs & Melvin (1961) showed that vascular contractions induced by noradrenaline are associated with increased calcium influx. Hudgins & Weiss (1968) have also shown that extracellular calcium is required for the contraction of smooth muscle induced by both noradrenaline and histamine. Cocaine has been shown to potentiate the responses to both histamine and noradrenaline in cat spleen (Innes unpublished), therefore experiments were designed to see if this potentiation involved changes in the utilization of extracellular calcium for contraction.

### 1. Noradrenaline

Experiments were done in 10 strips from 5 cats, each strip serving as its own control. Tissue calcium was first reduced by adding noradrenaline ($10^{-6}$ g/ml) to the bath containing a O-Ca EDTA solution until the contraction obtained was small. The bathing solution was then changed to Ca-Free, to remove the EDTA. A small contraction of constant height was obtained after a number of additions of noradrenaline to the bath. In five of the tissues 0.002 to 5.0 mM calcium was added to the bath in increments in the presence of noradrenaline ($10^{-6}$ g/ml). The addition of calcium caused a graded noradrenaline contraction (Fig. 11-A). The bath was then washed and the tissues allowed to relax in the presence of a

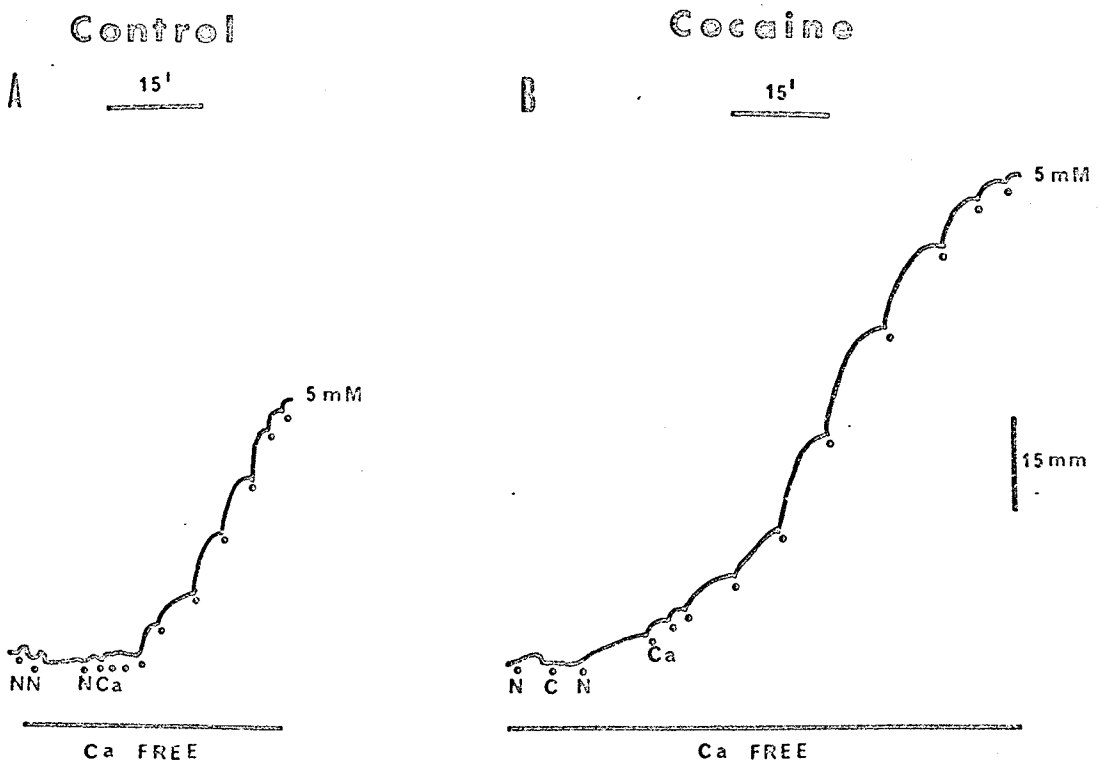


Fig. 11 The Effect of Cocaine on the Restoration of the Contraction to Noradrenaline in the Isolated Spleen Strip Bathed in a Calcium-Free Solution.

A- One spleen strip was exposed to cumulative concentrations of calcium (Ca, dots), 0.002 to 5.0 mM, in the presence of noradrenaline (N), $10^{-6}$ g/ml. B- 150 minutes later the same spleen strip was exposed to the same cumulative concentrations of calcium in the presence of noradrenaline (N), $10^{-6}$ g/ml, plus cocaine (C), $10^{-5}$ g/ml.

Ca-Free solution. Two and one-half hours later the same procedure was repeated in the presence of cocaine ($10^{-5}$ g/ml). Cumulative addition of calcium now caused a much larger graded noradrenaline contraction (Fig. 11-B). In five additional tissues from the same cats the order of treatment was reversed in order to provide a control for the effect of time; the tissues were treated with cocaine first and then followed by the control.

In order to eliminate the changes in the height of the dose response curve due to cocaine the contractile responses to each addition of calcium were calculated as a percentage of the total maximum after subtraction of the initial response to noradrenaline. These responses were then plotted against the calcium concentration in the bath. The effect of cocaine on the response to calcium restoration was determined by comparison of the calcium concentrations required to cause 50% of the maximum noradrenaline contraction. This concentration level was chosen because any change in the $ED_{50}$ is a measure of a shift in the dose response curve. Thus any change in the utilization of calcium will be reflected in a change in the $ED_{50}$. The results are shown in Fig. 12, and Table 8-A.

Cocaine shifted the calcium dose response curve to the left, and significantly reduced the amount of calcium necessary for 50% of the maximum noradrenaline contraction.

Karr (1966) showed that the maximum response to noradrenaline in spleen strips bathed in normal Krebs-Henseleit solution was not potentiated by cocaine. Therefore the same type of experiments as above were done in 8 strips from 4 cats with a larger dose of

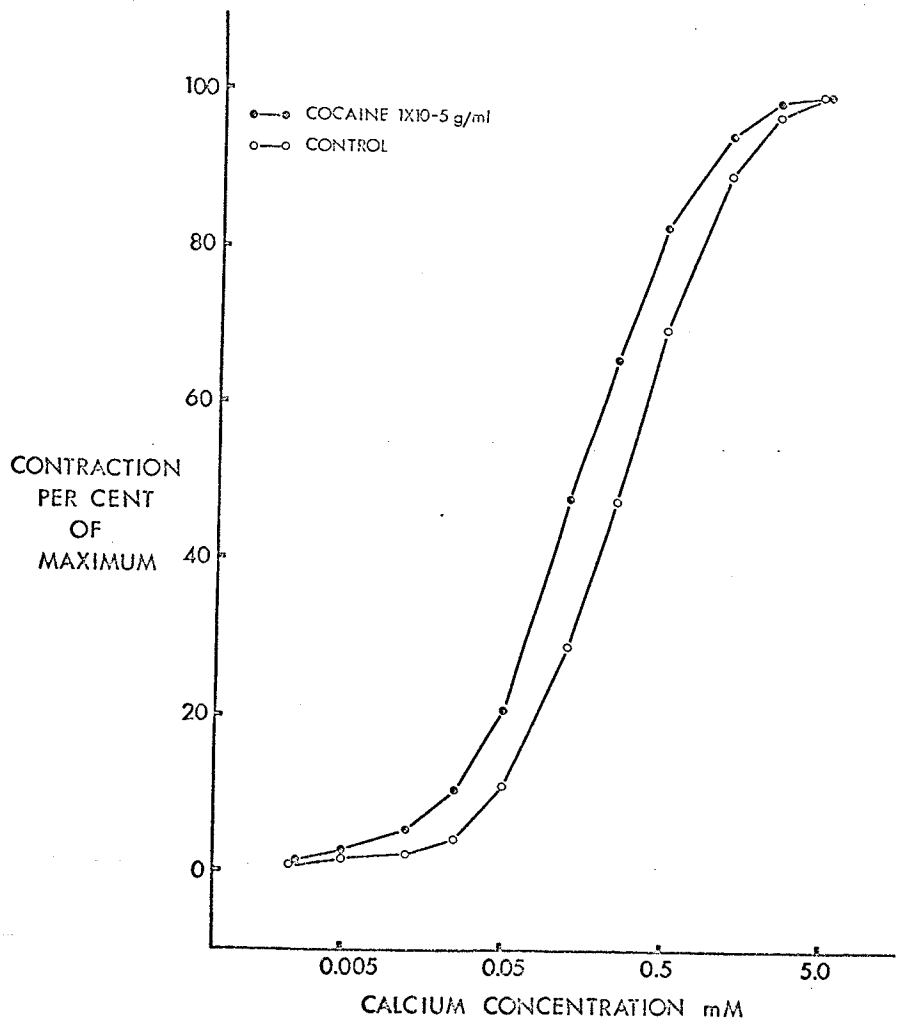


Fig. 12 The Effect of Cocaine, $10^{-5}$ g/ml, on the Restoration of the Contraction to Noradrenaline, $10^{-6}$ g/ml, by Cumulative Concentrations of Calcium in the Isolated Spleen Strip Bathed in a Calcium Free Solution.

Each point is the mean of 10 strips.

| Calcium Concentration mM | Mean Diff. ± S.E. Cocaine - Control % Maximum | P | |
|---|---|---|---|
| 0.002 | 0.4 ± 0.76 | N.S. | |
| 0.005 | 1.2 ± 1.20 | N.S. | |
| 0.012 | 3.3 ± 1.61 | N.S. | |
| 0.025 | 6.5 ± 2.85 | <0.05 | |
| 0.050 | 9.8 ± 3.09 | <0.05 | Paired Comparisons |
| 0.125 | 18.9 ± 4.52 | <0.01 | |
| 0.250 | 17.9 ± 4.36 | <0.01 | |
| 0.500 | 13.0 ± 4.58 | <0.05 | |
| 1.250 | 5.2 ± 1.42 | <0.01 | |
| 2.500 | 1.9 ± 0.68 | <0.05 | |

TABLE 8

CALCIUM CONCENTRATION NECESSARY TO PRODUCE 50% OF THE MAXIMUM RESPONSE TO NORADRENALINE AND HISTAMINE.

| | Treatment | Change of ED50 from Control Mean $\pm$ S.E. $\overline{mM}$ | P |
|---|---|---|---|
| A | Noradrenaline ($10^{-6}$g/ml) + Cocaine ($10^{-5}$g/ml) | -0.160 $\pm$ 0.042 (10) | $<0.005$ |
| B | Noradrenaline ($5\times10^{-5}$g/ml) + Cocaine ($10^{-5}$g/ml) | -0.022 $\pm$ 0.003 (4) | $<0.01$ |
| C | Histamine ($10^{-6}$g/ml) + Cocaine ($10^{-5}$g/ml) | +0.050 $\pm$ 0.030 (20) | $>0.05$ |
| D | Histamine ($10^{-6}$g/ml) + Cocaine ($3\times10^{-5}$g/ml) | -0.124 $\pm$ 0.042 (16) | $<0.02$ |

The Numbers in Parenthesis Represent the Number of Spleen Strips.

noradrenaline ($5 \times 10^{-5}$ g/ml) which could cause a maximum contraction. Any change in the calcium dose response curve therefore, would not be due to a change in the maximal response to noradrenaline.

Cocaine ($10^{-5}$ g/ml) shifted the calcium dose response curve to the left (Fig. 13), and significantly reduced the amount of calcium necessary for 50% of the maximum noradrenaline contraction (Table 8-B).

A further comparison was made between the amount of calcium necessary to cause 50% of the maximum respcnse to the larger and smaller concentrations of noradrenaline in the absence of cocaine. The mean $ED_{50}$ for calcium with noradrenaline ($10^{-6}$ g/ml) was $0.322 \pm 0.060$ mM which was significantly greater than the mean $ED_{50}$ for calcium with noradrenaline ($5 \times 10^{-5}$ g/ml), $0.094 \pm 0.007$, ($P < 0.05$).

2. Histamine

The effect of cocaine on the restoration of the contraction to histamine by calcium was tested to see if the alteration by cocaine of the requirement for less extracellular calcium was specific for noradrenaline.

Ten experiments as described above were done in 20 strips from 10 cats, each strip serving as its own control. In 10 preparations 0.002 to 5.0 mM calcium was cumulatively added to the bath in the presence of histamine ($10^{-6}$ g/ml); the bath was washed, and two and one-half hours later the same calcium concentrations were added to the bath in the presence of histamine ($10^{-6}$ g/ml) plus cocaine ($10^{-5}$ g/ml). In another 10 strips from the same cats the order of

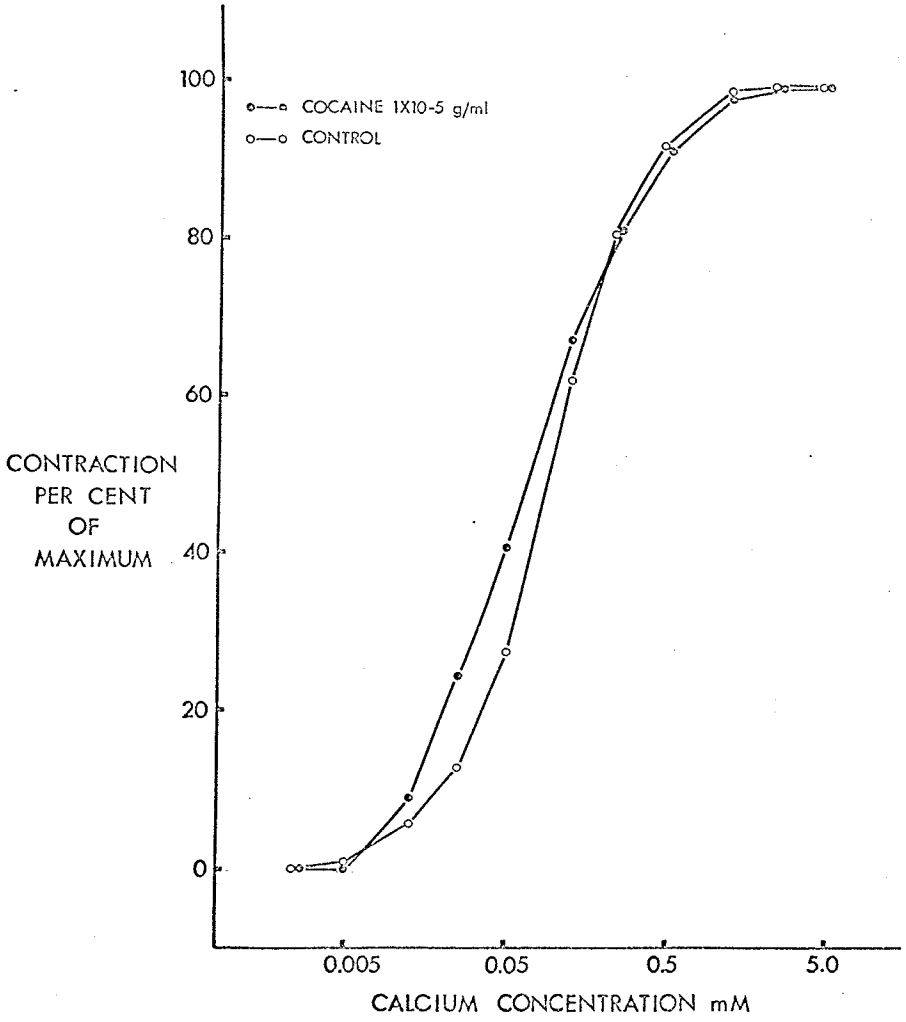


Fig. 13 The Effect of Cocaine $10^{-5}$ g/ml, on the Restoration of the Contraction to Noradrenaline, 5 x $10^{-5}$ g/ml, by Cumulative Concentrations of Calcium in the Isolated Spleen Strip Bathed in a Calcium-Free Solution.

Each point is the mean of 8 strips

| Calcium Concentration mM | Mean Diff. ± S.E. Cocaine - Control % Maximum | P | |
|---|---|---|---|
| 0.002 | 0.0 ± 0.02 | N.S. | |
| 0.005 | 0.9 ± 0.54 | N.S. | |
| 0.012 | 3.3 ± 2.32 | N.S. | |
| 0.025 | 11.7 ± 1.79 | <0.01 | |
| 0.050 | 13.2 ± 3.41 | <0.05 | Paired Comparisons |
| 0.125 | 5.2 ± 0.88 | <0.01 | |
| 0.250 | 0.7 ± 0.52 | N.S. | |
| 0.500 | 0.9 ± 0.77 | N.S. | |
| 1.250 | 1.0 ± 1.05 | N.S. | |
| 2.500 | 0.3 ± 0.33 | N.S. | |

treatment was reversed to control for the effect of time.

The results are shown in Fig. 14. Cocaine significantly shifted the lower and upper portions of the calcium dose response curve to the left, but the amount of calcium required for 50% of the maximum histamine contraction was not significantly increased (Table 8-C).

Further experiments were also done in 16 strips from 8 cats with the same dose of histamine but a larger dose of cocaine ($3 \times 10^{-5}$ g/ml). The results are shown in Fig. 15. Cocaine shifted the calcium dose response curve to the left, and significantly reduced the amount of calcium required for 50% of the maximum histamine contraction (Table 8-D).

3. Control Experiments

Control experiments were done to see if calcium would contract the isolated spleen strip in the presence or absence of cocaine. Experiments were done in 4 spleen strips from 4 cats. Tissue calcium was first reduced by bathing the spleen strips in a Ca-Free EDTA solution, and repeatedly exposing them to noradrenaline ($10^{-6}$ g/ml) until the contraction obtained was small. The bathing solution was then changed to Ca-Free, to remove the EDTA. The strips then exposed to cumulative concentrations of 0.002 to 5.0 mM calcium did not contract. When the same strips were exposed to the same calcium concentrations in the presence of cocaine ($10^{-5}$ g/ml), two responded with contractions of 4 and 5 mm after 5 mM calcium, the other two did not contract.

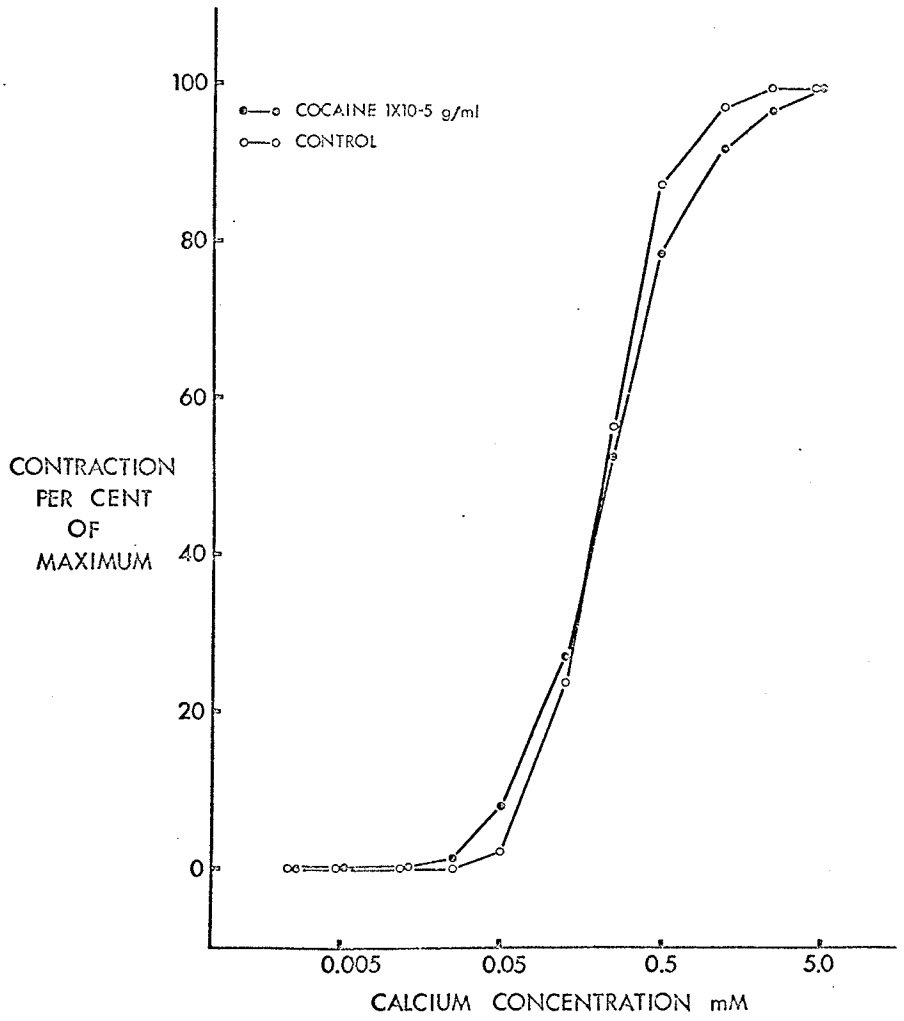


Fig. 14 The Effect of Cocaine, $10^{-5}$ g/ml, on the Restoration of the Contraction to Histamine, $10^{-6}$ g/ml, by Cumulative Concentrations of Calcium in the Isolated Spleen Strip Bathed in a Calcium-Free Solution.

Each point is the mean of 20 strips

| Calcium Concentration mM | Mean Diff. ± S.E. Cocaine - Control % Maximum | P | |
|---|---|---|---|
| 0.002 | 0.0 ± 0.00 | N.S. | |
| 0.005 | 0.1 ± 0.10 | N.S. | |
| 0.012 | 0.3 ± 0.22 | N.S. | |
| 0.025 | 1.4 ± 0.63 | <0.05 | |
| 0.050 | 5.8 ± 2.11 | <0.05 | Paired Comparisons |
| 0.125 | 3.2 ± 3.77 | N.S. | |
| 0.250 | 3.8 ± 5.17 | N.S. | |
| 0.500 | 8.9 ± 2.91 | <0.01 | |
| 1.250 | 5.6 ± 1.75 | <0.01 | |
| 2.500 | 2.9 ± 1.65 | N.S. | |

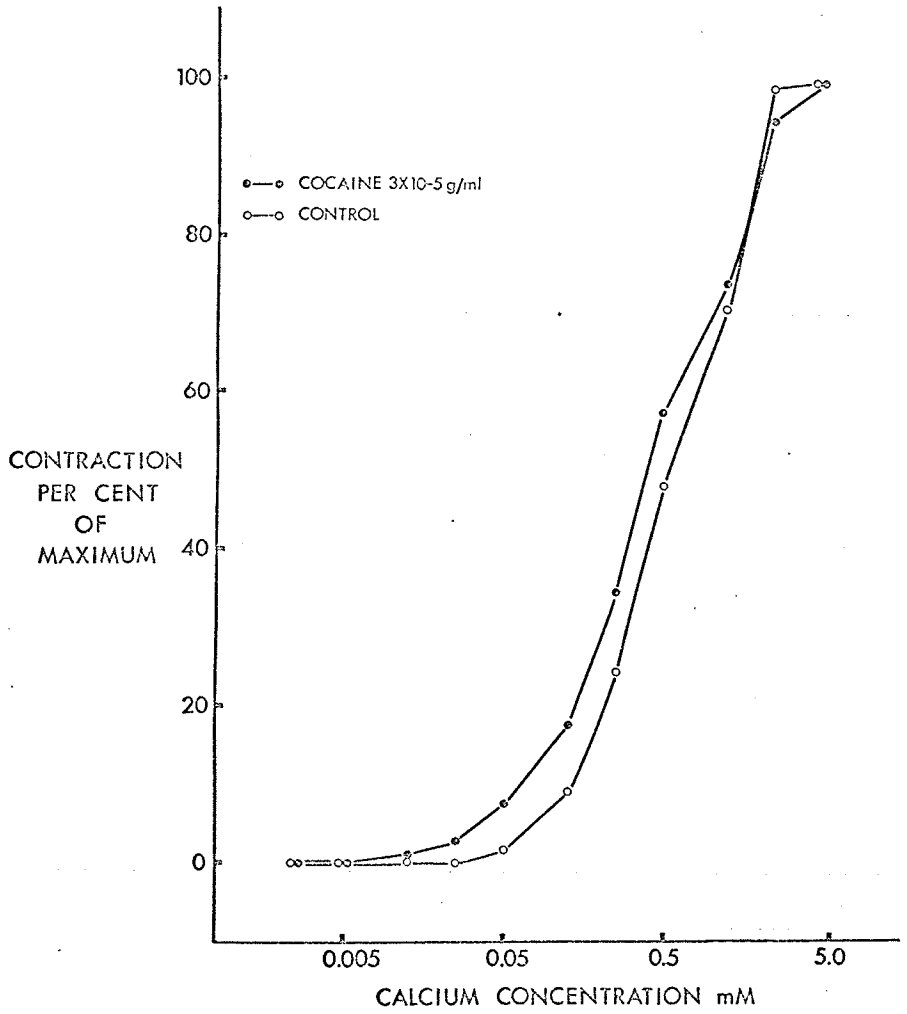


Fig. 15 The Effect of Cocaine, $3 \times 10^{-5}$ g/ml, on the Restoration of the Contraction to Histamine, $10^{-6}$ g/ml, by Cumulative Concentrations of Calcium in the Isolated Spleen Strip Bathed in a Calcium-Free Solution.

Each point is the mean of 16 strips

| Calcium Concentration mM | Mean Diff. ± S.E. Cocaine - Control % Maximum | P | |
|---|---|---|---|
| 0.002 | 0.0 ± 0.00 | N.S. | |
| 0.005 | 0.2 ± 0.22 | N.S. | |
| 0.012 | 1.0 ± 0.49 | N.S. | |
| 0.025 | 2.7 ± 1.34 | N.S. | |
| 0.050 | 5.5 ± 1.73 | <0.01 | Paired Comparisons |
| 0.125 | 8.6 ± 2.07 | <0.001 | |
| 0.250 | 10.3 ± 4.78 | <0.05 | |
| 0.500 | 9.6 ± 6.93 | N.S. | |
| 1.250 | 3.2 ± 4.33 | N.S. | |
| 2.500 | 4.3 ± 1.80 | <0.05 | |

C. PHARMACOLOGICAL ACTIONS OF DESMETHYLIMIPRAMINE

Desmethylimipramine (DMI) has been shown to potentiate the effects of noradrenaline in a wide variety of tissues. Sigg et al. (1963) showed that DMI enhanced the nictitating membrane contractions to both exogenous noradrenaline and preganglionic nerve stimulation. Bonaccorsi & Garattini (1966) showed that DMI potentiated the pressor response to noradrenaline, and antagonized the pressor response to tyramine in the pithed rat. Foster (1967) reported that DMI potentiated the relaxation by noradrenaline of the isolated guinea pig tracheal chain. DMI has also been shown to potentiate the responses to noradrenaline is isolated perfused renal artery, (Hrdina & Garattini 1966), in isolated rabbit atria (Matsu and Toda 1968). Pals & Masucci (1968) found that cocaine and DMI caused equal potentiation of the blood pressure response to $^{3}$H-noradrenaline in pithed rats, and concomitantly blocked the uptake of $^{3}$H-noradrenaline in the heart, adrenal gland and aorta. All these authors attributed the potentiating effects of DMI to the blockade of neuronal uptake of noradrenaline. However, Wastila & Maxwell (1966), found that DMI reduced the binding of noradrenaline by 61%, but did not potentiate the response to noradrenaline in isolated rabbit aortic strips.

DMI has also been shown to antagonize the responses to noradrenaline and adrenaline. Ursillo and Jacobson (1965) found that small concentrations of DMI potentiated, while relatively high concentrations of DMI antagonized, contractions of the isolated rat vas deferens due to noradrenaline. Hrdina and Garattini (1967) showed that DMI reduced adrenaline-induced contractions of the potassium

depolarized renal artery of the rat.

Many workers have reported that DMI inhibits the accumulation of catecholamines in various tissues; Iversen (1967), and Costa et al. (1966), in rat heart; Titus et al. (1966) in rat heart and kitten ventricle; Ross & Renyi (1967) in mouse brain.

Iversen et al. (1965) reported that DMI did not inhibit the uptake of $^{3}$H-noradrenaline in reserpine-treated rat hearts. However, these results are in marked contrast to those of Hamberger (1967), and Malmfors (1965), who showed histochemically that DMI blocked the neuronal uptake of noradrenaline in reserpine-treated rat brain, rat vas deferens, and rat iris.

Eisenfeld (1967) showed that DMI blocked 54% of the remaining uptake of $^{3}$H-noradrenaline in cocaine-treated isolated perfused rat hearts. He suggested that neuronal uptake was abolished by cocaine, and therefore the additional effect of DMI was extraneuronal. He further postulated that the reduction in extraneuronal uptake by DMI may be due to blockade of the adrenergic receptor sites, since DMI has adrenergic blocking properties.

Experiments in this section of results were primarily done to see if cocaine would potentiate the responses to noradrenaline in spleen strips where neuronal uptake had already been blocked by DMI. However, experiments were first done to see if the various properties of DMI described above apply to the isolated spleen strip.

1. The Effect of Desmethylimipramine and Cocaine on the Uptake of Noradrenaline in the Isolated Spleen Strip

It is generally accepted that reserpine blocks the uptake of

catecholamines at the nerve storage granule, and that cocaine and DMI block the uptake of noradrenaline at the axonal nerve membrane. The occurence of catecholamine uptake after reserpine has been reported quantitatively by Lindmar & Muscholl (1964), Iversen (1967), Glowinski et al. (1966), Dengler (1965) and histochemically, Malmfors (1965), Costa et al. (1966), and Hamberger (1967). Cocaine and DMI have been shown to block the uptake of catecholamines in reserpine-treated tissue, Hamberger (1967), Furchgott et al. (1963), and Malmfors (1965). Because of these findings reserpine-treated spleen strips were used to evaluate the blocking properties of DMI and cocaine on the uptake of noradrenaline.

Nine experiments were done with spleen strips cut from 9 cats given reserpine (1 mg/kg, 24 hours previously). One strip from each spleen was treated with DMI ($3 \times 10^{-5}$ g/ml) in 9 experiments, one strip with cocaine ($10^{-5}$ g/ml) in 8 experiments, and one strip with DMI ($3 \times 10^{-5}$ g/ml) plus cocaine ($10^{-5}$ g/ml) in four experiments. The experimental design is illustrated in Fig. 16.

Each strip was placed in a muscle bath and equilibrated in normal Krebs-Henseleit solution for one hour. The strips were then exposed to either DMI, cocaine, or DMI plus cocaine for 5 minutes. Noradrenaline ($5 \times 10^{-5}$ g/ml) was then added to the bath in the presence of these drugs and in one control tissue which did not receive any preliminary drug. The tissues were allowed to contract, and after 5 minutes all the drugs were washed from the bath. The strips were allowed to relax for 20 minutes, and then removed from the bath and assayed for catecholamines. One additional strip from each spleen

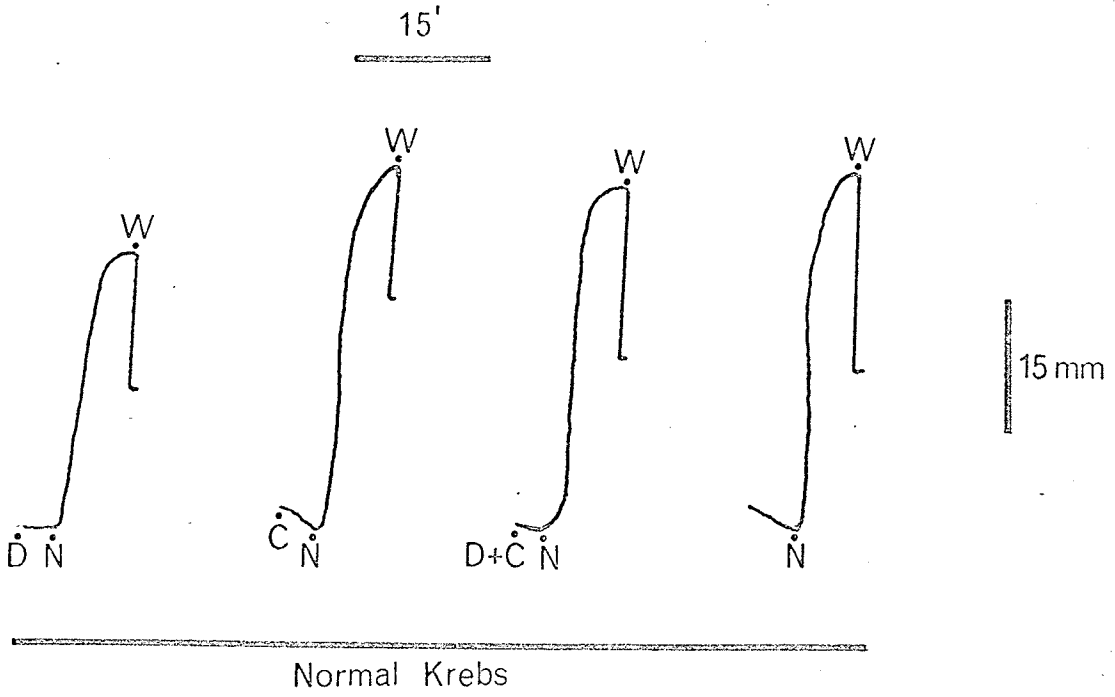


Fig. 16 The Effect of Desmethylimipramine and Cocaine on the Uptake of Noradrenaline in the Isolated Spleen Strip.

Four strips from the spleen of a cat given reserpine, 1 mg/kg, 24 hours before the experiment. One of the strips was treated with DMI (D), $3 \times 10^{-5}$ g/ml, one with cocaine (C), $10^{-5}$ g/ml, and a third with DMI $3 \times 10^{-5}$ plus cocaine $10^{-5}$ g/ml (D + C); the fourth strip was an untreated control. Noradrenaline (N), $5 \times 10^{-5}$ g/ml, was added to the baths of all four strips for 5 minutes; the baths were then washed (W).

which did not receive any drug treatment was assayed for catecholamines in order to test for completeness of depletion of catecholamines by reserpine. The results are summarized in Table 9.

The catecholamine uptake in the isolated spleen strip was significantly reduced by DMI, cocaine, or the combination of cocaine with DMI. The uptakes with DMI and with cocaine were not significantly different. Together DMI and cocaine caused a significantly greater reduction in uptake than did DMI alone, but not cocaine alone.

2. The Effect of Desmethylimipramine on the Response to Noradrenaline in Isolated Spleen Strips Bathed in Normal Krebs-Henseleit Solution

Six experiments were done to determine the effect of various concentrations of DMI on the response to noradrenaline in the spleen. Each experiment consisted of 4 strips from the same spleen. Each strip was equilibrated in normal Krebs-Henseleit solution for 60 minutes, then exposed to cumulative concentrations of noradrenaline $10^{-7}$ to $3 \times 10^{-4}$ g/ml. The bath was washed and the strips allowed to relax. Three of the 4 strips were then exposed to DMI $3 \times 10^{-8}$, $10^{-6}$, and $3 \times 10^{-5}$ g/ml for 5 minutes; the fourth served as a time control. All four strips were then tested with the previous concentrations of noradrenaline. The log dose-response curves obtained are shown in Fig. 17, and summarized in Table 10.

DMI $3 \times 10^{-8}$ and $10^{-6}$ g/ml significantly potentiated the responses to small concentrations of noradrenaline, but had no effect on the responses to large concentrations of noradrenaline. DMI $3 \times 10^{-5}$ g/ml significantly potentiated the responses to the lowest concentration of

TABLE 9

THE EFFECT OF DESMETHYLIMIPRAMINE AND COCAINE ON THE UPTAKE OF NORADRENALINE IN THE ISOLATED SPLEEN STRIP BATHED IN KREBS-HENSELEIT SOLUTION

Total Catecholamines, μg/gm of Tissue

| Cat # | A Control | B DMI ($3x10^{-5}$g/ml) | C Cocaine ($10^{-5}$g/ml) | D Cocaine ($10^{-5}$g/ml) + DMI ($3x10^{-5}$g/ml) | E No Loading |
|---|---|---|---|---|---|
| 1 | 0.726 | 0.316 | 0.294 | | 0.092 |
| 2 | 1.063 | 0.547 | 0.886 | | <0.002 |
| 3 | 0.446 | 0.258 | 0.116 | | <0.002 |
| 4 | 1.178 | 0.528 | | | <0.002 |
| 5 | 0.880 | 0.369 | <0.002 | | |
| 6 | 1.952 | 1.000 | 0.351 | 0.375 | 0.067 |
| 7 | 1.930 | 1.010 | 0.295 | 0.363 | <0.002 |
| 8 | 1.001 | 0.488 | 0.204 | 0.243 | |
| 9 | 1.075 | 0.566 | 0.269 | 0.251 | <0.002 |

Paired Comparisons

| | Mean Difference ± S.E. | P |
|---|---|---|
| A-B | 0.574 ± 0.08 | <0.001 |
| A-C | 0.832 ± 0.19 | <0.005 |
| A-D | 1.182 ± 0.23 | <0.02 |
| B-C | 0.267 ± 0.12 | >0.05 |
| B-D | 0.458 ± 0.10 | <0.02 |
| D-C | 0.028 ± 0.02 | >0.2 |

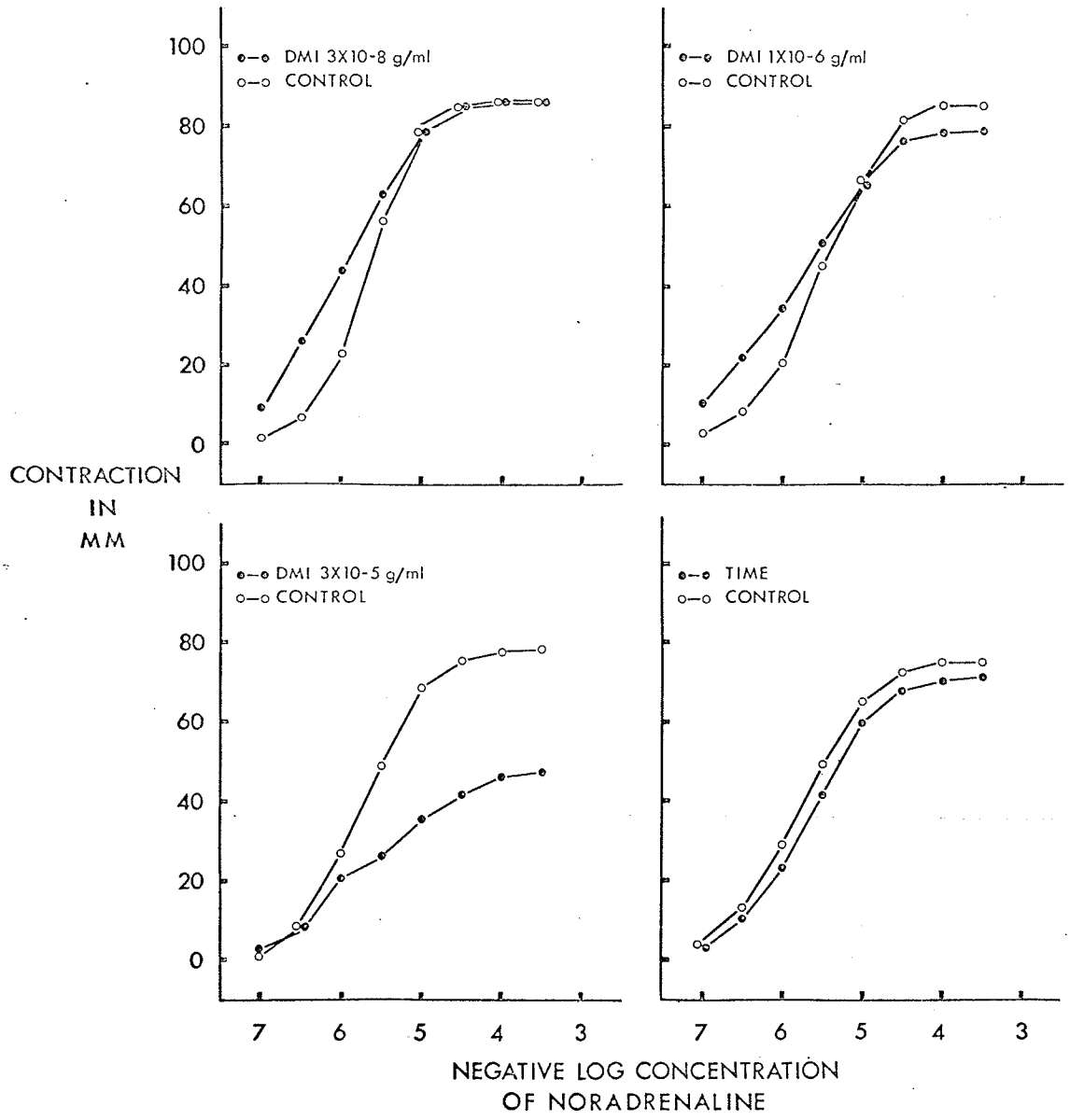


Fig. 17 The Effect of Desmethylimipramine on the Cumulative Log Concentration - Response Curves for Contraction of The Isolated Spleen Strip by Noradrenaline.

Each set of concentration-response curves is the mean of 6 experiments. Each experiment was done with 4 strips from the same spleen, 3 treated with DMI and one as a time control.

TABLE 10

THE EFFECT OF DESMETHYLIMIPRAMINE ON THE RESPONSE TO NORADRENALINE IN ISOLATED SPLEEN STRIPS BATHED IN NORMAL KREBS-HENSELEIT SOLUTION.

Difference From Control Contraction
Mean ± S.E.
mm

| Negative log of Noradrenaline Concentration g/ml | DMI $3x10^{-8}$g/ml | DMI $10^{-6}$g/ml | DMI $3x10^{-5}$g/ml | Time Control |
|---|---|---|---|---|
| 7.0 | 6.8 ± 2.21* | 7.2 ± 3.22 | 2.0 ± 0.37** | 0.6 ± 0.68 |
| 6.5 | 19.3 ± 4.38** | 13.8 ± 3.56* | 0.0 ± 3.95 | 2.9 ± 1.72 |
| 6.0 | 20.5 ± 7.21* | 13.8 ± 3.44* | 6.0 ± 7.84 | 6.1 ± 3.25 |
| 5.5 | 7.0 ± 4.58 | 5.7 ± 4.52 | 22.3 ± 8.56* | 7.9 ± 4.51 |
| 5.0 | 0.0 ± 3.22 | 1.2 ± 5.35 | 32.8 ± 7.29** | 5.2 ± 3.86 |
| 4.5 | 0.5 ± 2.53 | 5.7 ± 3.40 | 33.7 ± 8.40* | 4.4 ± 2.78 |
| 4.0 | 0.0 ± 2.52 | 4.7 ± 3.07 | 31.7 ± 8.30* | 4.9 ± 2.28 |
| 3.5 | 0.0 ± 2.52 | 4.3 ± 2.79 | 30.7 ± 8.42* | 3.5 ± 2.07 |

Each value in the table represents the mean difference from six strips obtained from different cats

* $P<0.05$
** $P<0.01$ Paired comparisons

noradrenaline, and significantly antagonized the responses to the five largest concentrations of noradrenaline.

3. The Effect of Desmethylimipramine on the Utilization of Extracellular Calcium for Contraction by Noradrenaline

The previous results showed that DMI ($3 \times 10^{-8}$ g/ml) potentiated the responses to noradrenaline ($10^{-6}$ g/ml). Experiments were therefore done to see if this potentiation involved changes in the requirement for extracellular calcium for contraction.

Experiments were done in 16 spleen strips from 8 cats, each strip serving as its own control. The experimental procedure was the same as described in Section B. In 8 strips 0.002 to 5 mM calcium was first added to the bath in increments in the presence of noradrenaline ($10^{-6}$ g/ml), and then in the presence of the same dose of noradrenaline plus DMI ($3 \times 10^{-8}$ g/ml). In 8 additional strips from the same cats the order of treatment was reversed to allow for the effect of time; the strip was treated with DMI first and then followed by the control. The contractile response to each addition of calcium was calculated as a percentage of the total maximum after subtraction of the initial response to noradrenaline. These responses were plotted against the calcium concentration in the bath. The results are shown in Fig. 18.

DMI did not alter the concentration of extracellular calcium necessary for contraction to noradrenaline. The calcium dose response curve in the presence of noradrenaline was not altered by DMI. The mean difference in calcium concentration necessary to produce 50 per cent of the maximum noradrenaline contraction between control

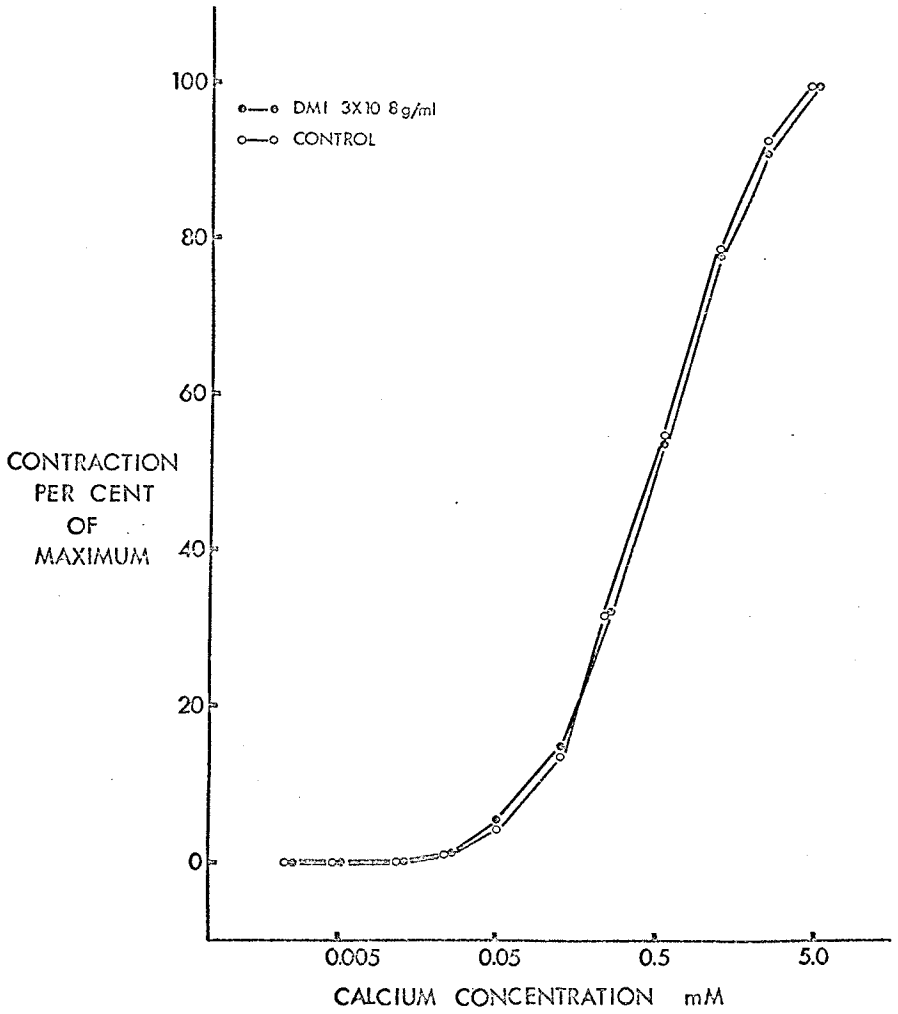


Fig. 18 The Effect of Desmethylimipramine 3 x $10^{-8}$ g/ml on the Restoration of the Contraction to Noradrenaline $10^{-6}$ g/ml by Cumulative Concentrations of Calcium in the Isolated Spleen Strip Bathed in a Calcium-Free Solution.

Each point is the mean of 16 strips.

and DMI treatment was not significantly different (0.034 $\pm$ 0.083 mM, $P > 0.06$). However, DMI did significantly potentiate the response to noradrenaline at calcium concentrations of 1.25, 2.5, and 5.0 mM. The mean differences in contraction height were 6.9 $\pm$ 2.8, 7.4 $\pm$ 3.1, and 8.1 $\pm$ 3.1 respectively, with a significance of $P < 0.05$.

4. The Effect of Desmethylimipramine on the Cocaine Potentiation of Noradrenaline in a Calcium-Free Solution

Six experiments were done to see if DMI would alter the potentiating effect of cocaine on noradrenaline in a calcium-free solution. Two strips from the same spleen were used in each experiment. Tissue calcium was first reduced in both strips by addition of noradrenaline ($10^{-6}$ g/ml) to the bath containing a O-Ca EDTA solution until the contraction obtained was small. The bathing solution was then changed to Ca-Free, to remove the EDTA. Both strips were then exposed to cocaine ($10^{-5}$ g/ml) in the presence of noradrenaline, ($10^{-6}$ g/ml). The baths were washed and the strips allowed to relax. Two and one-half hours later one strip was exposed to DMI ($3 \times 10^{-5}$ g/ml), and then cocaine ($10^{-5}$ g/ml), both in the presence of noradrenaline, (Fig. 19-A). The other strip served as a control and was exposed to cocaine in the presence of noradrenaline without DMI (Fig. 19-B).

The results are summarized in Table 11. DMI caused a small but significant increase in the noradrenaline contraction. In four additional strips this concentration of DMI alone did not cause a contraction. In the presence of this concentration of DMI, cocaine

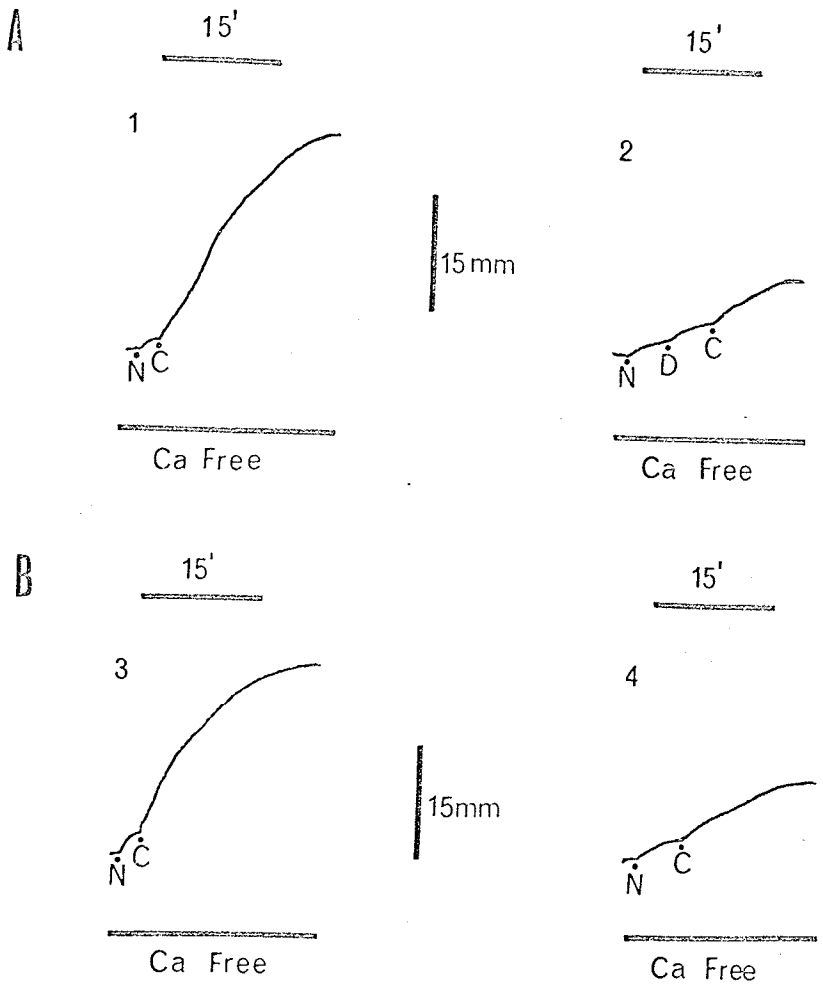


Fig. 19 The Effect of Desmethylimipramine on the Potentiation of Noradrenaline by Cocaine in the Isolated Spleen Strip Bathed in a Calcium-Free Solution.

Two spleen strips, A and B, from the same spleen A- Exposed to noradrenaline (N), $10^{-6}$ g/ml, and cocaine (C), $10^{-5}$ g/ml. 1. First response; 2. second response 150 minutes later in the presence of DMI (D), $3 \times 10^{-5}$ g/ml. B- Exposed to noradrenaline (N), $10^{-6}$ g/ml, and cocaine (C), $10^{-5}$ g/ml. 1. First response; 2. Second response 150 minutes later, without DMI.

TABLE 11

THE EFFECT OF DESMETHYLIMIPRAMINE ON THE POTENTIATION OF NORADRENALINE BY COCAINE IN A Ca-FREE SOLUTION.

| Treatment | Increase in Noradrenaline $10^{-6}$ g/ml Contraction Mean ± S.E. mm | | |
|---|---|---|---|
| | DMI ($3\times10^{-5}$ g/ml) | DMI ($10^{-6}$ g/ml) | DMI ($3\times10^{-8}$ g/ml) |
| DMI | 1.83 ± 0.33** (6) | 0.63 ± 0.20** (12) | 0.13 ± 0.12 (12) |
| Cocaine ($10^{-5}$ g/ml), After DMI | 3.83 ± 0.69** (6) | 4.54 ± 1.25** (12) | 3.13 ± 0.72*** (12) |
| Cocaine ($10^{-5}$ g/ml), Without DMI Control Response First | 10.83 ± 2.80* (6) | | |
| Cocaine ($10^{-5}$ g/ml), Without DMI Control Response Second | 3.66 ± 0.96* (6) | | |
| Cocaine ($10^{-5}$ g/ml), Control Responses Combined | | 5.21 ± 2.35* (12) | 2.96 ± 0.72** (12) |

* $P<0.05$
** $P<0.01$ Paired Comparisons
*** $P<0.001$

The Numbers in Parenthesis Represent the Number of Spleen Strips.

caused a further significant increase in the noradrenaline contraction.

In the control tissues both the first and second exposures to cocaine significantly increased the noradrenaline contraction, but the increase after the first exposure was significantly greater than after the second exposure, $P < 0.05$. In the control tissues the second exposure to cocaine caused only 33% of the initial cocaine response. In the presence of DMI the second exposure to cocaine caused only 22% of the initial cocaine response. However, the difference between these percentages was not statistically significant, $P > 0.05$.

Six additional experiments were done to see what effect DMI ($10^{-6}$, and $3 \times 10^{-8}$ g/ml) would have on the cocaine potentiation of noradrenaline in a calcium free solution. Four strips were cut from the same spleen, two were treated with DMI ($10^{-6}$ g/ml) and two with DMI ($3 \times 10^{-8}$ g/ml), each strip served as its own control. The tissues were bathed in a Ca-Free solution after tissue calcium was reduced as described above. In two strips the potentiation by cocaine ($10^{-5}$ g/ml) of the contraction to noradrenaline ($10^{-6}$ g/ml) was tested first. The baths were washed and the strips allowed to relax. Two and one-half hours later the potentiating effect of cocaine on noradrenaline was tested in the presence of DMI ($10^{-6}$ g/ml) in one strip and in the presence of DMI ($3 \times 10^{-8}$ g/ml) in the other (Fig. 20-A). In the other two strips the order of treatment was reversed; the strips were treated with DMI first and then followed by the control (Fig. 20-B). The results are summarized in Table 11.

In order to control for the effect of time the first and

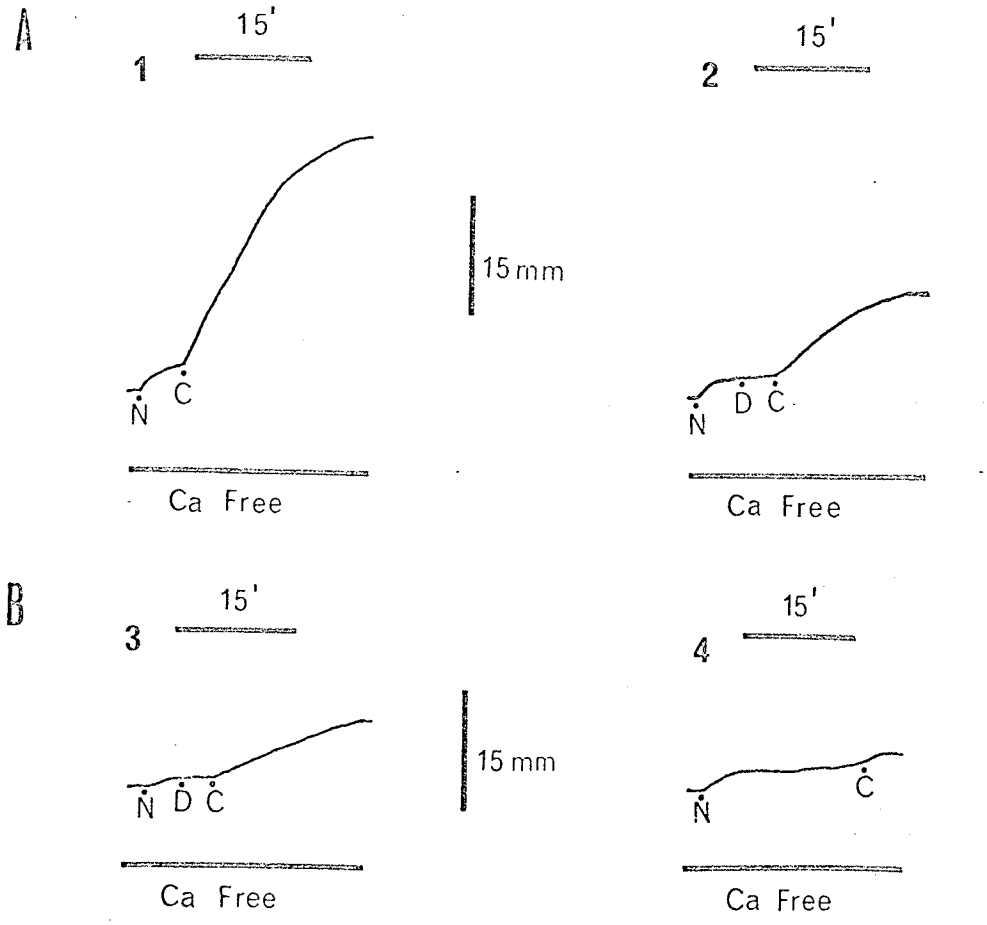


Fig. 20 The Effect of Desmethylimipramine on the Potentiation of Noradrenaline by Cocaine in the Isolated Spleen Strip Bathed in a Calcium-Free Solution.

Two strips, A and B, from the same spleen. A- Responses to noradrenaline (N), $10^{-6}$ g/ml, and cocaine (C), $10^{-5}$ g/ml. 1. First response; 2. Second response 150 minutes later in the presence of DMI $3 \times 10^{-8}$ g/ml.
B- Responses to noradrenaline (N), $10^{-6}$ g/ml, and cocaine (C), $10^{-5}$ g/ml;3. First response in the presence of DMI (D), $3 \times 10^{-8}$ g/ml; 4. Second response.

second responses were combined for the purpose of analysis. DMI ($10^{-6}$ g/ml) caused a very small but statistically significant increase (0.63 $\pm$ 0.20 mm) in the noradrenaline contraction; DMI ($3 \times 10^{-8}$ g/ml) had no effect. In four additional tissues DMI ($10^{-6}$ g/ml) alone did not cause a contraction. In the presence of either concentration of DMI cocaine caused a further significant increase in the noradrenaline contraction (4.54 $\pm$ 1.25 and 3.13 $\pm$ 0.72 mm), These increases in the height of the noradrenaline contraction were not significantly different from the increases caused by cocaine in the absence of DMI (5.21 $\pm$ 2.35 and 2.96 $\pm$ 0.72, $P > 0.8$ and $P > 0.9$).

D. THE EFFECT OF PROCAINE ON THE UTILIZATION OF EXTRACELLULAR AND INTRACELLULAR OR MEMBRANE BOUND CALCIUM BY NORADRENALINE FOR CONTRACTION

There have been conflicting reports on whether procaine potentiates the responses to catecholamines in smooth muscle. Armin et al. (1953) showed that procaine potentiated the adrenaline constriction of the rabbit ear artery, and Bentley (1965) showed that procaine potentiated the response to noradrenaline in isolated guinea pig vas deferens. Tainter & Chang (1927) found that procaine did not potentiate the heart rate and blood pressure responses to adrenaline in dogs and cats, and Nava-Rivera et al. (1967) found that procaine failed to potentiate, but antagonized the contractile response to noradrenaline in isolated aortic strips. Kalsner (1966) found that procaine failed to potentiate the responses to phenylephrine in rabbit aortic strips, but did block the uptake of phenylephrine.

Kuperman et al. (1968) and Weiss (1968) have shown that procaine caused an increased efflux of $^{45}Ca$ from frog sartorius muscle. Hudgins & Weiss (1968) showed that in rabbit aorta noradrenaline decreased the efflux of $^{45}Ca$, and that this effect was antagonized by procaine.

In view of these studies it was of interest to see if the action of procaine was similar to that found in the previous sections of results with cocaine. Experiments were therefore done to see if procaine would effect the amount of calcium necessary for contraction of the isolated spleen strip by noradrenaline.

Four experiments were done to see if procaine would potentiate the noradrenaline contraction in a Ca-Free solution. Two strips from the same spleen were used in each experiment. Tissue calcium was reduced in both strips by addition of noradrenaline ($10^{-6}$ g/ml) to the bath containing a O-Ca EDTA solution until the contraction obtained was small. The bathing solution was then changed to Ca-Free, to remove the EDTA. One strip was pretreated with procaine ($10^{-5}$ g/ml) for 5 minutes, after which noradrenaline ($10^{-6}$ g/ml) was added to the bath in the presence of the procaine (Fig. 21-A). The other strip was exposed to the same concentration of procaine after the contraction to noradrenaline had reached a plateau (Fig. 21-B). Procaine did not effect the noradrenaline contraction in either case. Both strips were then exposed to cocaine ($10^{-5}$ g/ml), which significantly increased the noradrenaline contraction by $10.0 \pm 2.1$ mm ($P < 0.05$), and $10.3 \pm 2.5$ mm ($P < 0.05$) respectively.

Eight additional experiments were done to see if procaine would alter the restoration of the noradrenaline contraction by calcium. Experiments were done in 8 spleen strips from 4 cats; each strip was used as its own control. The experimental procedure was the same as described in Section B. In four strips 0.002 to 5.0 mM calcium was added to the bath in increments in the presence of noradrenaline ($10^{-6}$ g/ml), and then in the presence of the same concentration of noradrenaline plus procaine ($10^{-5}$ g/ml). In four additional strips from the same cats the order of treatment was reversed to control for any effect of time; the strip was tested with procaine first and then without. The results are shown in

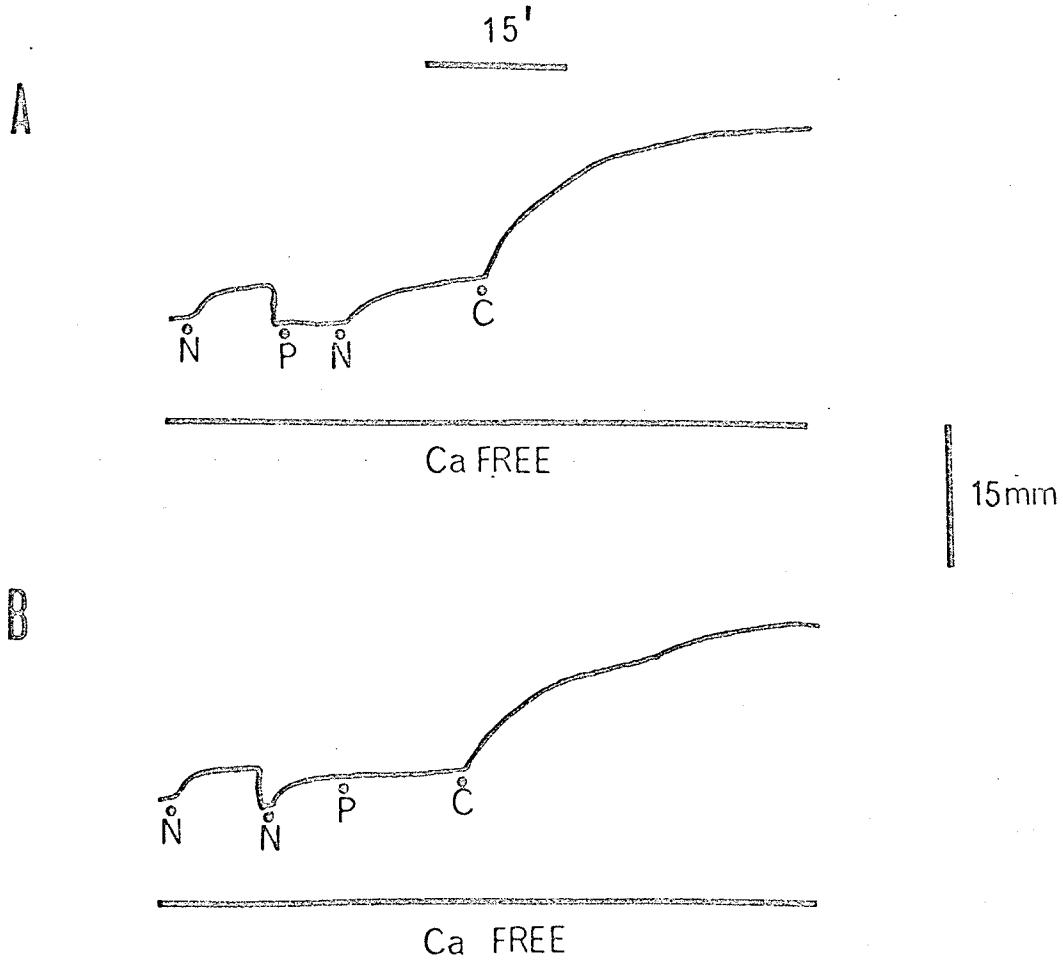


Fig. 21 The Effect of Procaine on the Response to Noradrenaline in the Isolated Spleen Strip Bathed in a Calcium-Free Solution.

Two spleen strips, A and B, from the same spleen. A- Exposed to noradrenaline (N), $10^{-6}$ g/ml, before and in the presence of procaine (P), $10^{-5}$ g/ml, and then cocaine (C), $10^{-5}$ g/ml. B- Exposed to noradrenaline (N), $10^{-6}$ g/ml, followed by procaine (P), $10^{-5}$ g/ml, and cocaine (C), $10^{-5}$ g/ml.

Fig. 22.

The calcium dose response curve in the presence of noradrenaline was not altered by procaine. The mean difference in calcium concentration necessary to produce 50 per cent of the maximum noradrenaline contraction between control and procaine treatment was not significantly different ($0.002 \pm 0.052$ mM $P > 0.9$). Procaine also did not significantly alter the response to noradrenaline at any of the calcium concentrations.

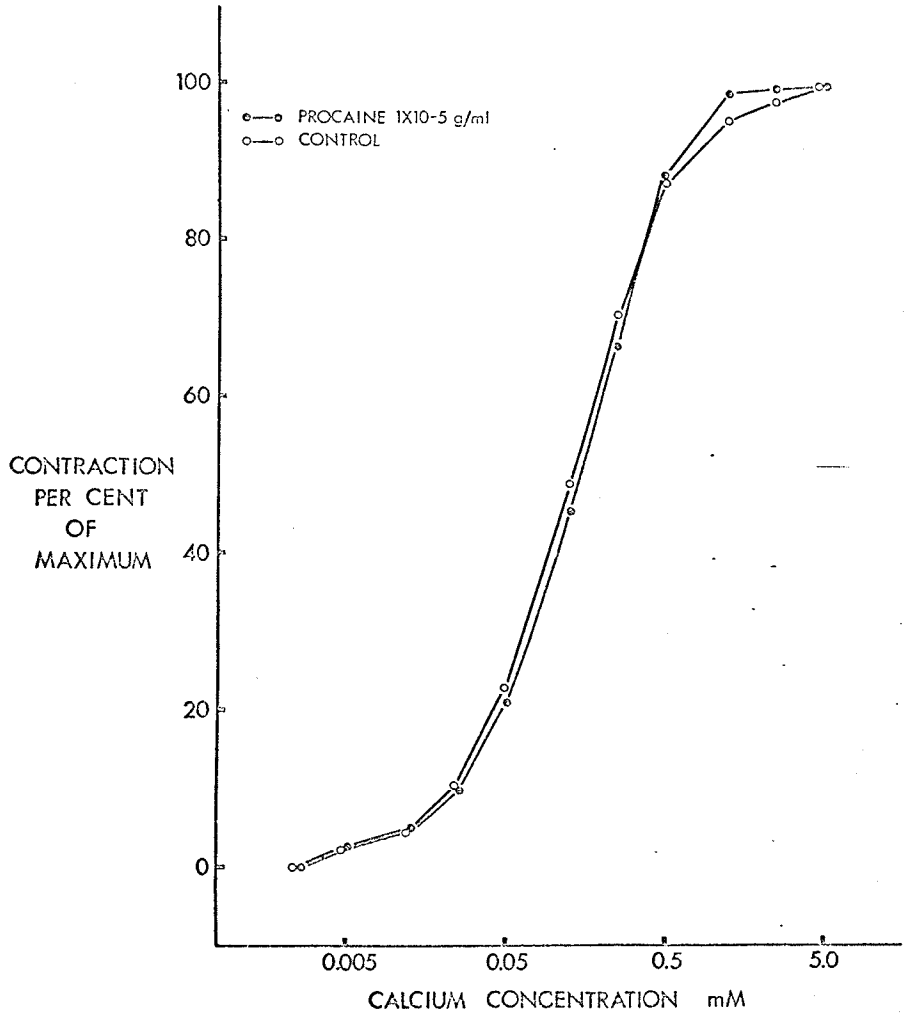


Fig. 22 The Effect of Procaine $10^{-5}$ g/ml on the Restoration of the Contraction to Noradrenaline, $10^{-6}$ g/ml, by Cumulative Concentrations of Calcium in the Isolated Spleen Strip Bathed in a Calcium-Free Solution.

Each point is the mean of 8 strips

# DISCUSSION

The results suggest that there is an alternative to the uptake hypothesis as the sole explanation for cocaine-induced supersensitivity. It is proposed that cocaine has a post-receptor site of action, in that it facilitates both the release or displacement of bound calcium, and the utilization of this calcium by the contractile elements. This proposal is supported by experiments showing that cocaine potentiates the contraction of the isolated spleen strip by noradrenaline or strontium in the absence of extracellular calcium, and that this potentiation is antagonised by calcium chelation. It was also found that in the presence of cocaine less extracellular calcium was required for the contraction of the spleen strip by either noradrenaline or histamine. This supports the view that either more calcium has become available to the contractile elements, or that the contractile elements can utilize calcium more efficiently.

## A. THE EFFECT OF COCAINE ON THE UTILIZATION OF BOUND CALCIUM BY NORADRENALINE OR HISTAMINE

The results clearly show that cocaine potentiates the contractile response to noradrenaline in the isolated spleen strip in the absence of extracellular calcium. There are a number of possible explanations for this potentiation. First, cocaine could increase the concentration of noradrenaline at the receptor by either releasing endogenous noradrenaline from, or blocking the uptake of exogenous noradrenaline by storage sites in the nerve endings. Second, cocaine could increase the affinity of the receptor for noradrenaline. The third and most likely explanation is that cocaine in some way makes a bound store of calcium more available for the contraction of the spleen strip by noradrenaline.

1. Release of Noradrenaline

Maengwyn-Davies & Kopanyi (1966) found that large concentrations of cocaine ($10^{-3}$ g/ml) caused the contraction of the isolated rabbit aortic strip by the release of endogenous catecholamines. Daniel & Wolowyk (1966) showed that very large concentrations of cocaine caused the contraction of the isolated uterus, by a direct action on the smooth muscle. However, in our experiments, where the maximum concentration of cocaine used was $10^{-5}$ g/ml, much less than in the above experiments on aortic strip and uterus, cocaine never caused contraction of the spleen strip bathed in a calcium-free solution, but did potentiate the small contraction caused by noradrenaline. Depletion of the catecholamine stores by reserpine did not effect this potentiation by cocaine. Kirpekar & Wakade (1968) showed that extracellular calcium is necessary for the release of noradrenaline from post-ganglionic sympathetic nerves in the cat spleen. Therefore the potentiation by cocaine in the absence of extracellular calcium can not be due to a direct effect of cocaine on the smooth muscle, or the release of endogenous catecholamines.

2. Potentiation of the Maximum Response to Noradrenaline by Cocaine

Cocaine increased the maximum response to noradrenaline in the spleen strip bathed in a calcium-free solution. This finding differs from the observations made by Karr (1966) on the spleen strip bathed in the presence of normal calcium in which cocaine did not increase the maximum response to noradrenaline. However, the maximum contraction to noradrenaline under the conditions of severe calcium lack is much less than that seen when normal calcium is present. It is presumed that in severe calcium lack

noradrenaline causes contraction by releasing a tightly bound calcium store. If cocaine makes this calcium store more available to release by noradrenaline it could account for the increased maximum contraction.

It is unlikely that the increased maximum contraction is due to an increase in the noradrenaline concentration at the receptor as the result of blockade of neuronal uptake by cocaine, as the maximum contraction was obtained at concentrations of noradrenaline $3 \times 10^{-5}$ g/ml and $10^{-4}$ g/ml; any further increase in the concentration of noradrenaline did not cause a further contraction, but cocaine did.

Another possible explanation for the change in maximum response to noradrenaline is that cocaine increases the affinity of the adrenergic receptor for noradrenaline. Tuttle (1968) showed that calcium facilitates the combination of noradrenaline with the alpha adrenergic receptor. If cocaine released bound calcium into the vicinity of the adrenergic receptor the released calcium could possibly promote its binding with noradrenaline. Davidson & Innes (unpublished) found that there were no spare receptors for noradrenaline in a spleen strip bathed in a solution containing normal calcium. This implies that all the receptors are occupied by noradrenaline in order for noradrenaline to elicit a maximum response. However, it is possible that the maximum response to noradrenaline under conditions of severe calcium lack is not due to occupancy of 100% of the receptors. If this is true cocaine could conceivably promote the utilization of more receptors by noradrenaline by freeing calcium from an unavailable store and thus making calcium available to the receptors. However, this explanation is unlikely in view of the fact that cocaine itself does not contract the spleen strip bathed in a calcium-free solution. If it were to release

bound calcium one would expect it to elicit a contraction. Results were also obtained which showed that cocaine potentiated the contraction of the spleen strip caused by strontium which is presumed not to act on the adrenergic receptor.

3. The Role of Bound Calcium in the Contraction of the Spleen Strip to Noradrenaline

At least two binding sites for calcium in addition to an extracellular site have been postulated for smooth muscle by a number of authors Hinke (1965), Daniel (1965), Hurwitz (1967b) and Hudgins & Weiss (1968). They suggested that there is a calcium store which is loosely bound to the outside of the membrane and which is easily chelated by Na-EDTA, and a tightly bound calcium store at an intracellular site, which may possibly be bound to the inside of the membrane and which is not chelated by EDTA. Hinke (1965) and Hudgins & Weiss (1968) suggested that noradrenaline can utilize all three sources of calcium for contraction of smooth muscle. These theories however, are based on indirect evidence, and the exact nature and location of multiple calcium storage sites has not been determined. Until there is a complete understanding of the mechanism of contraction of smooth muscle, and the exact role of calcium in this mechanism is determined, the experimental evidence must be regarded as circumstantial and the interpretation of the action of drugs on smooth muscle will remain speculative. Therefore the results obtained in this study will be discussed in the light of the current theories, but it must be kept in mind that these theories have not been firmly established.

The results show that noradrenaline utilizes bound calcium for contraction

of the spleen strip. This was shown by experiments where Na-EDTA caused relaxation of the noradrenaline contraction in a calcium-free solution, and repeated Na-EDTA administration reduced the magnitude of a subsequent noradrenaline contraction. These results suggest that Na-EDTA can chelate loosely and tightly bound calcium after they have been released by noradrenaline from their binding sites. The failure of Na-EDTA to relax the very small second noradrenaline contraction may be attributed to the complete absence of loosely bound calcium, and the release of only a very small amount of bound calcium, which therefore does not diffuse out of the cell and can not be chelated by Na-EDTA. Subsequent exposure of the spleen strip to normal Krebs-Henseleit solution replaced more bound calcium than was originally present in the tissue after the tissue had been bathed for two hours in a calcium-free solution containing EDTA. This was demonstrated by the much larger contraction in response to noradrenaline in a calcium-free solution after calcium replacement, than in a calcium-free solution after two hours of exposure to a O-Ca EDTA solution.

4. The Role of Bound Calcium in the Potentiation of Noradrenaline by Cocaine

Cocaine was shown to potentiate the response to noradrenaline in a calcium-free solution. In these experiments tissue calcium was reduced by repeated noradrenaline administration in the presence of a O-Ca EDTA solution. Therefore all the extracellular and loosely bound calcium were probably removed from the spleen. The inability of Na-EDTA to abolish completely this small noradrenaline contraction indicates that the calcium store must be very tightly bound and very difficult to exhaust. The

remaining small noradrenaline contraction must therefore be due to the release of calcium from this tightly bound store. However, in the presence of cocaine noradrenaline caused a much greater contraction, which seems to be due to the ability of noradrenaline to release more calcium from this bound store. Therefore cocaine in some way makes this calcium more available for release by noradrenaline, thereby potentiating the contractile response to noradrenaline without cocaine itself causing a contraction. Inhibition by EDTA of the cocaine-induced potentiation of the response to noradrenaline indicates that the calcium which is released from the binding site diffuses out of the cell along its concentration gradient and then is chelated by Na-EDTA.

The repeated administration of Na-EDTA antagonized the potentiation of noradrenaline by cocaine in a calcium-free solution, and greatly reduced the magnitude of subsequent responses to noradrenaline and cocaine. However, these responses were partially restored by replacing tissue calcium. These results suggest that the ability of cocaine to potentiate the responses to noradrenaline in a calcium-free solution is dependent on the size and location of the bound calcium store. The reduction in the effect of cocaine by repeated Na-EDTA administration may be due to the removal of all the loosely bound calcium, leaving only the tightly bound calcium, which is much more difficult for noradrenaline to release, or may be due to a depletion or reduction in the amount of tightly bound calcium.

The results obtained with Ca-EDTA demonstrate that the inhibition of the potentiation of noradrenaline by cocaine in a calcium-free solution is due to calcium chelation and not to an unspecific depressant effect of EDTA on the spleen strip. Na-EDTA caused relaxation of the noradrenaline-cocaine

contraction, but Ca-EDTA caused further contraction. This unexpected contractile effect of Ca-EDTA is most likely due to some free calcium which was present as an impurity in the Ca-EDTA and not bound to the EDTA, since the same concentration of Ca-EDTA did not contract the spleen strip when it was bathed in normal Krebs-Henseleit solution. Daniel & Irwin (1965) obtained similar results in the isolated uterus. They found that Ca-EDTA can restore contraction by supplying calcium to the smooth muscle which had previously been depleted of calcium to the point of having lost its contractility; this effect was antagonized by Na-EDTA.

5. Inhibition by Diazoxide of the Potentiation of Noradrenaline by Cocaine

Wohl et al. (1967) showed that Diazoxide is a non-competitive inhibitor of noradrenaline in the rat aorta bathed in a solution with normal calcium content. In another study Wohl et al. (1968) showed that Diazoxide competitively antagonized the restoration by calcium of the contraction caused by noradrenaline in the rat aorta bathed in a calcium-free solution. They suggest that a calcium pool is linked to the alpha adrenergic receptor somewhere in the chain of events between activation of the receptor and the contractile elements, and that Diazoxide prevents the utilization of this calcium for contraction. Their explanation does not adequately explain the non-competitive inhibition of the contraction to noradrenaline, and the competitive inhibition of the calcium restoration of the noradrenaline contraction. These results may also be interpreted in view of the findings of Tuttle (1968) that calcium facilitates the combination of noradrenaline with the receptor. Diazoxide could therefore prevent calcium from facilitating this drug-receptor combination, and thus act at a site other than

the noradrenaline receptor, causing a non-competitive antagonism. Any increase in the noradrenaline concentration therefore would not overcome the antagonism. Diazoxide in the same way would competitively antagonize the calcium restoration of the response to noradrenaline in a calcium-free solution, where the magnitude of the response is related to the calcium concentration. Therefore any increase in the calcium concentration would overcome the Diazoxide antagonism.

We have shown that in a calcium-free solution Diazoxide antagonizes the potentiation of the contraction to noradrenaline by cocaine, but did not antagonize the response to noradrenaline itself. The failure to antagonize noradrenaline can be explained by the absence of extracellular calcium available to facilitate combination of noradrenaline with the receptor; under these experimental conditions antagonism of the potentiating action of cocaine would be explained by interference by Diazoxide in some way with the effect of cocaine in making a bound store of calcium more available for noradrenaline to release. Therefore two sites of action of Diazoxide are proposed, one where it blocks the action of extracellular calcium so that the combination of noradrenaline with the receptor is impaired; and another site where it blocks the action of cocaine on a bound calcium store.

These results might also be explained on the basis of Diazoxide chelating calcium. However, the chemical structure of Diazoxide makes this unlikely, and there is no chemical evidence for such a reaction.

6. The Contraction of the Spleen Strip by Strontium in a Calcium-Free Solution and its Potentiation by Cocaine

Strontium has been shown to cause contraction of smooth muscle

(Daniel, 1963). It has also been shown that strontium can substitute for calcium in the contraction of smooth muscle (Sperelakis, 1962; Daniel, 1963; Bohr, 1964), and in skeletal muscle (Frank, 1962; Caldwell & Walster, 1963). It has been suggested by both Frank (1962) and Daniel, (1965) that strontium substitutes for calcium by displacing bound intracellular calcium.

Our results show that strontium caused equivalent contractions of normal and reserpine-treated spleen strips bathed in a calcium-free solution. Therefore the contraction was not due to the release of stored noradrenaline. Cocaine potentiated these responses to strontium but had a much greater effect in the reserpine-treated strips than in the normal strips. These results are fully consistent with the hypothesis that strontium causes contraction of the isolated spleen strip by releasing bound calcium, and that this bound calcium is much more easily released in the presence of cocaine. The finding that cocaine potentiated the contraction in response to strontium much less in reserpine-treated than normal strips can be explained on the basis of the different procedures used for the removal of tissue calcium. In the normal strips tissue calcium was reduced by bathing the strip in a O-Ca EDTA solution and repeatedly exposing the strip to noradrenaline until the contraction obtained was small. However, to avoid replenishment of any of the noradrenaline stores in the reserpine-treated strip, the strips were not exposed to noradrenaline and tissue calcium was reduced only by bathing the strip in a O-Ca EDTA solution for two hours. It is also possible that less bound calcium was present in the reserpine-treated strip. Carrier & Shibata (1967) showed that reserpine depleted tissue calcium in rabbit aorta. Therefore cocaine may be able to make more bound calcium available for noradrenaline to release in the normal than the reserpine-treated strips.

These results lend further support to a post-receptor action of cocaine i.e. on bound calcium, as they did not involve uptake or release of noradrenaline, and the adrenergic receptor was not involved in any way.

7. Histamine

The ability of histamine to contract the isolated spleen strip was greatly reduced in the absence of extracellular calcium, and when tissue calcium was reduced by repeated exposure to histamine in the presence of a O-Ca EDTA solution. Cocaine failed to potentiate these very small histamine contractions, but did potentiate the contraction of these same strips in response to noradrenaline. These results are in agreement with the findings of Hudgins & Weiss (1968) who showed that histamine depends mainly on extracellular and loosely bound calcium for the contraction of smooth muscle, while noradrenaline can utilize a tightly bound calcium store. Our results have shown that in the absence of extracellular and loosely bound calcium cocaine potentiated noradrenaline but not histamine. Noradrenaline can use intracellular or tightly bound membrane calcium for contraction but histamine can not. Therefore cocaine may make this calcium store more available; this calcium is then utilized by noradrenaline and not by histamine.

The results showing that noradrenaline potentiated the contraction of the isolated spleen strip by histamine in a calcium-free solution indicate that noradrenaline releases bound calcium which is then available for histamine to utilize for contraction. It was assumed that if cocaine enables noradrenaline to release more bound calcium, then the noradrenaline potentiation of the histamine contraction should be greater in tissues which

were treated with cocaine. However the histamine contractions in the presence of noradrenaline were not larger in the cocaine treated strips than in the control strips. This was an unexpected finding but might be due to having reached the maximal contractile response of the spleen strip in the presence of cocaine.

The effect of Na-EDTA on the histamine contractions is also puzzling. Na-EDTA caused relaxation of the histamine response in the cocaine-treated strips, but caused a further contraction superimposed on the response to histamine in the control strips. One possible explanation might be that a greater amount of calcium was released by histamine in the cocaine-treated strips than in the control strips. This large amount of calcium might be enough to cause some diffusion out of the cell, where it would be chelated by Na-EDTA. On the other hand the small amount of calcium released in the absence of cocaine would not be sufficient to diffuse out of the cell and therefore could not be chelated by the Na-EDTA. This explanation is not wholly satisfactory as the potentiation of the histamine response by noradrenaline should have then been greater in the cocaine-treated than in the control strips, which did not occur. This may have been due to the maximum contractile response of the cocaine-treated strips having been reached. Further experiments with smaller noradrenaline concentrations might elucidate these discrepencies.

The Na-EDTA contraction of the spleen strip which was not treated with cocaine could be the result of the release of a small amount of bound calcium. Daniel & Irwin (1965) found that EDTA contracted the uterus in the absence of extracellular calcium. They suggested that this contraction was the result of the removal of calcium from the surface of the cell membrane

which in some way caused the liberation of calcium from an intracellular binding site. The results obtained here therefore might be explained on the same basis.

## B. THE EFFECT OF COCAINE ON THE UTILIZATION OF EXTRACELLULAR CALCIUM BY NORADRENALINE OR HISTAMINE FOR CONTRACTION OF THE SPLEEN STRIP.

It is well established that noradrenaline and histamine utilize extracellular calcium for the contraction of smooth muscle (Briggs & Melvin, 1961; Waugh, 1965; Hinke, 1965; Hudgins & Weiss, 1968). Our results are consistent with this view, and have shown that both noradrenaline and histamine utilize extracellular calcium for contraction of the isolated spleen strip. In addition, cocaine altered the utilization of this calcium for contraction. Less extracellular calcium was necessary for an equivalent noradrenaline or histamine contraction in the presence of cocaine than in the control.

### 1. Noradrenaline

The maximum height of the calcium dose-response curve in the presence of the submaximal dose of noradrenaline ($10^{-6}$ g/ml) was potentiated by cocaine. These changes in height were eliminated by plotting the dose response-curves as a percentage of the total maximum response to noradrenaline at the largest calcium concentration. Therefore when cocaine shifted the calcium dose-response curve to the left it was the result of less extracellular calcium being utilized for contraction, and not the result of a change in the maximum height of contraction. It was found that the amount of calcium necessary to produce 50% of the maximum response to noradrenaline

was less after cocaine treatment.

The maximum height of the calcium dose-response curve in the presence of the larger concentration of noradrenaline ($5 \times 10^{-5}$ g/ml) was not altered by cocaine. However, the calcium dose-response curve was shifted to the left by cocaine and the amount of calcium required for 50% of the maximum response to noradrenaline was less after treatment with cocaine than in the control.

The changes in the calcium restoration curves for noradrenaline may be accounted for in three ways. First, cocaine could alter the membrane permeability and thus increase the entrance of extracellular calcium into the cell, so that for a given calcium concentration noradrenaline would cause a greater contraction in the presence of cocaine by virtue of more calcium entering the cell and reaching the contractile elements. Hurwitz (1962) showed that cocaine antagonized the responses to acetylcholine and potassium in guinea pig ileum. He suggests that this antagonism is the result of membrane stabilization which prevents depolarization and the influx of extracellular calcium which is necessary for contraction. However, Hurwitz has no direct evidence for cocaine preventing calcium influx upon depolarization. Unlike the guinea pig ileum the contraction of the spleen strip by acetylcholine is potentiated and not antagonized by cocaine (Innes, unpublished). In addition if cocaine stabilized the membrane and prevents the influx of calcium in the spleen strip, more and not less extracellular calcium should be required for contraction by noradrenaline.

The second explanation may be that cocaine effects the contractile elements in some way so that they become more sensitive to calcium. Noradrenaline then would cause an equivalent contraction in the presence of

less calcium.

The third and most likely explanation is that in the presence of cocaine noradrenaline releases more bound calcium, thereby making less extracellular calcium necessary for contraction. The experiments done in the absence of extracellular calcium showed that the contraction of the isolated spleen strip by noradrenaline was due to the release of bound calcium. The large concentration of noradrenaline ($10^{-5}$ g/ml) caused a larger contraction than the smaller concentration of noradrenaline ($10^{-6}$ g/ml), presumably by releasing more bound calcium. The results obtained with restoration of extracellular calcium showed that less extracellular calcium was required for 50% of the maximum response to noradrenaline ($5 \times 10^{-5}$ g/ml) than to noradrenaline ($10^{-6}$ g/ml) (see page 78). These results suggest that less extracellular calcium is required for contraction by the larger concentration of noradrenaline because more bound calcium is released. The effect of cocaine would be explained by its ability to cause noradrenaline to release more bound calcium; thus less extracellular calcium would be required for contraction.

These results are consistent with the findings of Hinke (1966) who showed that segments of ventral tail artery from rats made hypertensive with desoxycorticosterone required less extracellular calcium for contraction to noradrenaline or potassium than did similar preparations from normotensive rats. He suggested that the "hypertensive"artery performed more work than the "normotensive" artery, and that the increased responsiveness was due to an increased efficiency in either calcium utilization during excitation-contraction coupling or in the contractile mechanism itself. Our results do not preclude an action of cocaine on the contractile elements.

Such an effect might facilitate the utilization of calcium by the contractile elements and thus less extracellular calcium would be required for an equivalent contraction in the presence of cocaine. Bohr (1964) proposed a similar mechanism for the potentiation of adrenaline by desoxycorticosterone in isolated rabbit aortic strips. He suggested that the steroid makes more calcium available from bound sites within the cell and therefore potentiates the response to adrenaline. In either of these studies, the findings may readily be explained by any one of the three possible mechanisms discussed here.

2. Histamine

A larger concentration of cocaine was required to change the amount of calcium required for the contraction of the spleen strip by histamine than by noradrenaline. This larger cocaine concentration ($3 \times 10^{-5}$ g/ml) shifted the calcium dose-response curve to the left and less calcium was necessary to cause 50% of the maximum contraction to histamine in the presence of cocaine than in the control. Davidson & Innes (unpublished) have shown that a larger concentration of cocaine is required to potentiate histamine than noradrenaline contractions of the spleen strip. The present results are consistent with the idea of small cocaine concentration being specific for the potentiation of noradrenaline, and the larger concentration of cocaine being unspecific, potentiating both noradrenaline and histamine.

The calcium restoration curves for histamine are somewhat puzzling. Less extracellular calcium was necessary for an equivalent histamine contraction in the presence of cocaine ($3 \times 10^{-5}$ g/ml) than in the control. The results in section A (see Fig. 8) showed that cocaine did not potentiate

the contraction of the spleen strip by histamine in a calcium-free solution because histamine can not utilize tightly bound calcium for contraction. It is therefore difficult to explain these results on the basis that less extracellular calcium is necessary for a histamine contraction in the presence of cocaine because cocaine allows histamine to release more bound calcium. However, if cocaine were to alter the contractile elements in some way so that they become more sensitive to calcium, histamine would cause equivalent contraction of the spleen strip in the presence of less calcium. This would account for the cocaine potentiation of the calcium restoration of the histamine contraction where extracellular calcium is available for contraction and for the failure of cocaine to potentiate the histamine contraction in a calcium-free solution where extracellular calcium is unavailable.

## C. PHARMACOLOGICAL EFFECTS OF DESMETHYLIMIPRAMINE

Desmethylimipramine has been shown to have a dual effect on the responses to noradrenaline in the vas deferens and renal artery of the rat (Ursillo & Jacobson, 1965; Hrdina & Garattini, 1967). We have confirmed these findings, using full dose-response curves for the contraction of the isolated spleen strip by noradrenaline in a normal calcium solution. Small concentrations of DMI potentiated the contraction to small and medium concentrations of noradrenaline, but did not alter the maximum contraction to noradrenaline. However, when we extended the study to the effect of DMI in a calcium-free medium, these small concentrations of DMI failed to potentiate the responses to noradrenaline in a calcium-free solution, or to alter the amount of extracellular calcium utilized for contraction of

the spleen strip (see Fig. 18 & 20). Therefore the potentiating effect of DMI may be entirely due to an increased noradrenaline concentration at the receptor as the result of blockade of neuronal uptake by DMI.

Large concentrations of DMI ($3 \times 10^{-5}$ g/ml) antagonized the responses to noradrenaline in a normal calcium solution. The dose-response curve for noradrenaline was shifted to the right and the maximum height greatly reduced, which indicates a non-competitive type of antagonism. These large concentrations of DMI, however, failed to antagonize the contraction to noradrenaline in a calcium-free solution. These results suggest that the antagonism is not due to an action on the alpha adrenergic receptor. Our observations agree with those of Hrdina & Garattini (1967) who showed that the contraction of the isolated rat renal artery by potassium was relaxed by DMI, and that this effect was antagonized by calcium. They also showed that contractions of the artery due to either noradrenaline or calcium were antagonized by DMI. From these results they concluded that the antagonism of noradrenaline by DMI was due to the prevention of the utilization of extracellular calcium for contraction. Our results concur with this view, since DMI antagonized the responses to noradrenaline in the presence of extracellular calcium but failed to antagonize the response to noradrenaline in the absence of extracellular calcium. The mechanism of noradrenaline antagonism by DMI could be further elucidated by seeing if DMI ($3 \times 10^{-5}$ g/ml) prevents calcium from restoring the contraction due to noradrenaline in spleen strips bathed in a calcium-free solution.

It is well established that DMI blocks the uptake of catecholamines into storage sites in sympathetic nerve endings, but there are conflicting reports on its ability to block the uptake of noradrenaline in reserpine-

treated tissues (Iversen et al., 1965; Hamberger, 1967; Malmfors, 1965). Our results show that DMI effectively blocks the uptake of noradrenaline in the isolated reserpine-treated spleen strip. These observations are in contrast to those of Iversen et al. (1965)on the reserpine-treated rat heart, where DMI did not block noradrenaline uptake. However, the results are in agreement with those of Malmfors (1965), and Hamberger (1967), who showed histochemically that DMI blocked the uptake of noradrenaline and alpha-methyl noradrenaline in reserpine-treated rat brain, vas deferens and iris.

We further showed that in the reserpine-treated cat's spleen a larger concentration of DMI than of cocaine was required to block the uptake of noradrenaline (see Table 9). Together DMI and cocaine caused a greater reduction in noradrenaline uptake than did DMI alone, but not cocaine alone. These results indicate that while DMI effectively blocks the uptake of noradrenaline it is not as potent an inhibitor as cocaine. The molecular weights of cocaine and DMI are very similar, therefore the comparison of potency can be made on a molar basis. Our results are similar to those obtained by Hamberger (1967) who found that the neuronal uptake of noradrenaline was blocked equally by cocaine and DMI in reserpine-treated rat cerebral cortex or vas deferens. However, our results are in contrast to those obtained in tissues from animals which were not treated with reserpine. On a molar basis DMI has been shown to be more effective than cocaine in blocking the uptake of noradrenaline in rat heart (Iversen, 1967); in rat adrenals, heart and aorta (Pals & Massucci, 1967); and in mouse cerebral cortex (Ross & Renyi, 1967). Eisenfeld et al. (1967) found that DMI was able to further block the uptake of noradrenaline in isolated rat hearts

where uptake was already blocked with a very large concentration of cocaine. The difference between our results and those cited above could be due to differences in the techniques used in the measurement of noradrenaline uptake, in reserpine treatment, or in the tissues used in the various studies. Further experiments to measure uptake of tritiated noradrenaline in normal spleen might resolve these differences.

Cocaine effectively potentiated the responses to noradrenaline in spleen strips bathed in a calcium-free solution where the neuronal uptake of noradrenaline had already been blocked by DMI. The uptake experiments showed that DMI effectively blocked the uptake of noradrenaline in the reserpine-treated spleen strip bathed in a normal calcium solution. However, the experiments where cocaine potentiated the response to noradrenaline in the presence of DMI, were done in a calcium-free solution. Various workers have reported that lack of calcium does not influence uptake. Iversen (1966) showed that the uptake of noradrenaline by the rat heart was not affected when calcium was omitted from the perfusion fluid. Kirpekar &Wakade (1968) showed that calcium was not required for the uptake of noradrenaline by the perfused cat spleen. Hamberger (1967) showed histochemically that calcium was not required for the uptake of alpha-methyl noradrenaline by the isolated reserpine-treated vas deferens. In addition, Malmfors (1965) and Titus et al. (1966) showed that the blockade of noradrenaline uptake by DMI was competitive, providing evidence that DMI and noradrenaline act at the same uptake site. If calcium is not required for the uptake of noradrenaline, and both DMI and noradrenaline act at the same site, it is very unlikely that the uptake of noradrenaline or its blockade by DMI in the isolated spleen strip would be altered by

bathing in a calcium-free solution.

In our uptake experiments DMI ($3 \times 10^{-5}$ g/ml) blocked about 50% of the uptake of a very large concentration of noradrenaline ($5 \times 10^{-5}$ g/ml). However, a much smaller concentration of noradrenaline ($10^{-6}$ g/ml) was used in the experiments in which we demonstrated potentiation of noradrenaline by cocaine in the presence of DMI. Malmfors (1965) and Titus et al. (1966) showed that DMI was much more effective in blocking the uptake of small concentrations of noradrenaline than large concentrations of noradrenaline (in rat iris, rat heart, and kitten ventricle). Hamberger (1967) showed that DMI ($3 \times 10^{-5}$ g/ml) completely abolished the uptake of alpha-methyl noradrenaline ($10^{-6}$ g/ml), in the rat brain and vas deferens. Therefore cocaine potentiated the contraction to noradrenaline in the spleen strip bathed in a calcium-free solution where it is presumed that the neuronal uptake of the dose of noradrenaline used was virtually abolished by a large concentration of DMI.

## D. THE EFFECT OF PROCAINE ON THE POTENTIATION OF NORADRENALINE BY COCAINE

The experiments done with procaine showed that it did not effect the utilization of either extracellular or bound calcium for the contraction of the spleen strip by noradrenaline. Procaine failed to potentiate the noradrenaline contraction of the spleen strip bathed in a calcium-free solution and did not alter the subsequent cocaine potentiation. Procaine also failed to alter the contraction of the spleen strip in the presence of normal calcium or the effect of calcium restoration of the noradrenaline contraction in a calcium-free solution.

Hudgins & Weiss (1968) showed that procaine inhibited the contraction

of the rabbit aortic strip by noradrenaline but not by potassium. They further showed that noradrenaline decreased the efflux of $^{45}Ca$ and that this effect was inhibited by procaine. On the basis of these findings they suggested that procaine prevents noradrenaline from releasing bound calcium. Our results are contrary to these findings and indicate there is no such action of procaine on the isolated spleen strip. It is presumed that the contraction of the isolated spleen strip in a calcium-free solution by noradrenaline and its potentiation by cocaine are due to the release of bound calcium; procaine in this situation was without effect.

# SUMMARY

1. Noradrenaline caused a small contraction of the isolated spleen strip bathed in a calcium-free solution; this contraction was greatly potentiated by cocaine. It is suggested that noradrenaline causes contraction by releasing bound calcium, and that cocaine potentiates by making more bound calcium available for release by noradrenaline.

2. Potentiation of noradrenaline by cocaine in a calcium-free solution was not due to an increase in the noradrenaline concentration at the receptor as the result of either the release of endogenous noradrenaline or the blockade of uptake of exogenous noradrenaline. Cocaine potentiated the contraction to noradrenaline in a calcium-free solution where the noradrenaline stores were depleted by reserpine. The maximum noradrenaline contraction in the absence of extracellular calcium was increased by cocaine, and cocaine potentiated the responses to noradrenaline after neuronal uptake was blocked by desmethylimipramine.

3. Na-EDTA antagonized the potentiation of noradrenaline by cocaine in a calcium-free solution, and greatly reduced the magnitude of subsequent responses to noradrenaline and cocaine. These results indicate that Na-EDTA chelates the calcium once it is released from its binding site and diffuses out of the cell along its concentration gradient.

4. Diazoxide did not alter the contraction of the spleen strip to noradrenaline in a calcium-free solution, but antagonized the potentiation of noradrenaline by cocaine. It is suggested that Diazoxide interferes with or prevents cocaine from making more bound calcium available for the release by noradrenaline.

5. Strontium caused equivalent contractions of normal and reserpine-treated spleen strips bathed in a calcium-free solution. Cocaine potentiated these responses but had a much greater effect in the reserpine-

treated strips than in the normal strips. It is suggested that strontium causes the contraction of the spleen strip in the absence of extracellular calcium by releasing or displacing bound calcium, and cocaine in some way facilitates the release of this calcium.

6. The response of the spleen strip to histamine was greatly reduced in the absence of extracellular calcium, and was not potentiated by cocaine. However, noradrenaline did potentiate the histamine contraction in a calcium-free solution. The failure of cocaine to potentiate histamine was attributed to the inability of histamine to release bound calcium, and the noradrenaline potentiation attributed to the release of bound calcium which was then utilized by histamine for contraction.

7. Cocaine altered the utilization of extracellular calcium by noradrenaline or histamine for contraction of the spleen strip. Less extracellular calcium was required for an equivalent noradrenaline or histamine contraction in the presence of cocaine than in the control. It was found that the amount of calcium necessary to produce 50% of the maximum response to noradrenaline or histamine was reduced by cocaine. These results indicate that cocaine increased the membrane permeability to calcium, allowed more bound calcium to be released, or altered the contractile elements in some way so that they became more sensitive to calcium.

8. Desmethylimipramine blocked the uptake of noradrenaline in the isolated reserpine-treated spleen strip, but a larger concentration of DMI than of cocaine was required. Together DMI and cocaine caused a greater reduction in noradrenaline uptake than did DMI alone, but not cocaine alone.

9. Depending on the concentration used, desmethylimipramine either

potentiated or antagonized the responses to noradrenaline in the spleen strip. Small concentrations of DMI potentiated the noradrenaline contraction of the spleen strip in the presence of normal calcium, but not in a calcium-free solution. These small DMI concentrations did not alter the amount of extracellular calcium utilized for contraction of the spleen strip by noradrenaline. Large concentrations of DMI non-competitively antatonized the contraction of the spleen strip by noradrenaline in the presence of normal calcium. However, these large concentrations of DMI did not antagonize the response to noradrenaline in the absence of extracellular calcium. It is suggested that the potentiating effect of DMI in the presence of extracellular calcium is due to the blockade of neuronal uptake, while the antagonism is due to the prevention of the utilization of extracellular calcium by noradrenaline for contraction.

10. Procaine did not effect the utilization of either extracellular or bound calcium for contraction of the spleen strip by noradrenaline. Procaine also did not alter the potentiation of noradrenaline contraction of the isolated spleen strip by cocaine in a calcium-free solution.

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