STUDIES ON SUPERSENSITIVITY OF SMOOTH MUSCLE IN THE CAT SPLEEN

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ABSTRACT

A variety of agents and procedures is known which enhance the response of smooth muscle to noradrenaline. Many of these impair the tissue catecholamine uptake processes, and the currently popular uptake hypothesis assumes this to be the cause of associated supersensitivity. This thesis reports the results of experiments with cocaine, chronic denervation and reserpine on responses of the isolated cat spleen, in which pre-receptor, receptor, and post-receptor events in the stimulus-reponse sequence are examined.

Experiments bearing on the importance of impaired uptake in the development of supersensitivity are reported. Substances which competitively antagonize impairment of noradrenaline uptake by cocaine fail to protect against its potentiating action, whereas phentolamine, a drug with little effect on uptake, prevents cocaine-induced potentiation. Submaximal steady-state responses to noradrenaline, wherein the agonist has equilibrated with uptake sites, are nevertheless potentiated by cocaine. Submaximal excitatory responses to isoproterenol are potentiated by cocaine, yet uptake of this amine is insignificant. These data show a lack of functional correlation between changes in uptake and tissue sensitivity. Because phentolamine is known to be an alpha receptor antagonist, and because cocaine bears so little structural resemblance to the agonist it affects, it is suggested that its interaction with cocaine may involve allosteric transitions affecting the affinity of the alpha receptor.

Possible changes in receptor affinity are examined with alpha receptor antagonists. These agents have affinity for the receptors, but lack efficacy. In contrast to the agonists, their concentration is little affected by the usual disposal mechanisms. Both chronic denervation and cocaine enhance non-equilibrium receptor block by phenoxybenzamine. Reserpine has no effect. It is suggested that cocaine and chronic denervation may both increase the affinity of alpha receptors for phenoxybenzamine.

Maximum responses and calcium utilization for contraction are studied as a measure of changes in efficacy and post-receptor processes. Cocaine and denervation have no effect on the maximum response to a full agonist (noradrenaline), but reserpine causes it to increase. Since there are spare receptors for this agonist, it is concluded that reserpine acts at a post-receptor level on factors which normally limit the maximum contraction. The maximum response to a partial agonist (isoproterenol), or to noradrenaline after removal of spare receptors with phenoxybenzamine, is limited by the effectiveness of the drug-receptor complex. Neither chronic denervation nor cocaine increase the maximum under these conditions, indicating that efficacy as a property of the activated receptors is unchanged. Calcium utilization by tissues depleted of their calcium and stimulated with noradrenaline is not significantly changed by denervation, cocaine or reserpine. Our failure to cause complete abolition of the noradrenaline response in depleted tissues suggests that some calcium remained, and renders these results inconclusive.

It is suggested that cocaine and denervation cause super-

sensitivity by a similar mechanism which is independent of their effects on uptake. Increased affinity of the alpha receptors for substances normally acting on them is suggested as an explanation consistent with the results. Reserpine causes supersensitivity by a different mechanism involving post-receptor events which are response-limiting.

WITH LOVE

TO MY PATIENT AND UNDERSTANDING WIFE

SANDRA

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INTRODUCTION

A. HISTORICAL

Supersensitivity is a term which has been widely used to describe increased responses to stimuli in excitable tissues in a variety of circumstances. As such it is not a definitive term, and offers no information about the underlying mechanisms. This and other related terms, e.g. augmentation, exaggeration (of a response) or potentiation (of a stimulus), will be used interchangeably in this thesis without any implied distinction in meaning.

From the review by Cannon & Rosenblueth (1949), it is apparent that two classical observations in the middle of the last century prompted much of the early investigation of increased sensitivity in excitable tissues. The first of these was the report by Budge in 1855 that the cat iris responded to asphyxia with marked dilation after its sympathetic nerve supply had degenerated. Eight years later, Philipeaux & Vulpian observed that the dog tongue contracted to stimulation of associated parasympathetic nerves (chorda tympani) when its normal nerve supply (hypoglossal) had degenerated. These initial observations were limited to smooth and striated muscles, but it is now apparent that any type of excitable tissue may be made supersensitive by removing its functional innervation. Thus supersensitivity has been observed to adrenaline in the sympathetically denervated heart (Burrett, 1940) to strychnine and acetylcholine in efferent spinal neurones which have had their central connections interrupted by hemisection of the spinal cord (Cannon & Haimovici, 1939), to acetylcholine in autonomic ganglia after preganglionic section (Cannon & Rosenblueth, 1936), to pilocarpine in submaxillary

salivary glands after preganglionic parasympathetic nerve section (Pierce & Gregersen, 1937), and to adrenaline after postganglionic sympathetic nerve section (Simeone & Maes, 1939). The almost universal occurence of this response to loss of innervation prompted Cannon in 1939 to formulate his law of denervation which states that "When in a series of efferent neurones a unit is destroyed, an increased irritability to chemical agents develops in the isolated structure or structures, the effects being maximal in the part directly denervated."

Supersensitivity thus initially appeared to be a phenomenon related to surgical denervation. However, since 1910, when Fröhlich & Loewi reported that cocaine increased the pressor and mydriatic responses to small doses of adrenaline, many drugs have been discovered which increase the sensitivity of excitable tissues.

Of the many types of tissue and response which show supersensitivity, probably none is more complex, more thoroughly investigated and least understood than the smooth muscle response to adrenergic stimuli. Evidence in recent years has indicated the existence in sympathetically innervated smooth muscle of a complex mechanism for the uptake and storage of catecholamines. The finding that many procedures which interfere with this process also increase sensitivity to catecholamines has led to a widely accepted "uptake hypothesis" which holds that uptake and sensitivity are functionally related. This thesis is concerned with supersensitivity to adrenergic stimuli, and with the adequacy of the uptake hypothesis to account for it.

B. EARLY OBSERVATIONS OF SUPERSENSITIVITY IN SMOOTH MUSCLE

In 1903, Anderson reviewed the early literature on denervated* smooth muscles and began a thorough investigation of his own. He noted that the early observations of Budge on the denervated iris were repeated by other physiologists of his time (Tuwin, 1881; Kowalewsky, 1886). However, these workers were all limited in their ability to interpret the phenomenon by inadequate knowledge of the way in which the iris is normally activated. Budge and Tuwin both postulated a weakening of the sphincter muscle of the iris to explain the exaggerated dilation. Kowalewsky suggested that the superior cervical ganglion exerted an inhibitory influence on the dilator muscle, and that the effect of this loss was seen after denervation. Surminsky (1869) believed that the effect was due to a contracture of the smooth muscle after section of its motor nerves.

Anderson's own study (1905) showed that the paradoxical response of the iris was due to a direct effect of stimulating the dilator muscle. In an animal at complete rest the denervated iris was no more dilated than the intact control, thus ruling

* 'Denervation' and 'chronic denervation' are used interchangeably throughout this thesis to mean section of postganglionic nerves to an effector several days before its sensitivity is tested. This is to be distinguished from 'chronic decentralization' or prior section of the preganglionic nerves to an effector. Section of the nerves at the time of the experiment is never implied in this thesis.

out contracture of the radial muscle or a weakened sphincter. Excitement dilated the pupil even when the tone of the sphincter was maintained at a high level by eserine or strong light, thus further disproving the notion of a weakened sphincter. If the preganglionic sympathetic nerve was cut but the ganglion left intact, the paradoxical dilation still occurred, though to a lesser extent, thus arguing against an inhibitory influence by the ganglion. Anderson concluded that it was due to an increased excitability of the smooth muscle which was denervated. Elliot (1905) observed that adrenaline provoked exaggerated dilation of the pupil. He also found increased sensitivity of denervated pilomotor muscles, retractor penis and nictitating membrane, and made this generalization: "This then is true for all muscles thrown into contraction by adrenaline, that after decentralization, and still more clearly after denervation, they contract in the presence of adrenaline alike with greater irritability and persistence."

Thus supersensitivity to adrenergic stimuli was shown to be a potential property of several sympathetically innervated tissues. This has provided the framework for investigation of the still unanswered question: when the nerves to a tissue degenerate what are the changes which result in an increased response to stimulation? Many changes occur in tissue after denervation, but only some of these changes will necessarily be functionally related to development of supersensitivity. Mere demonstration of a temporal correlation between two such events cannot establish causality.

C. OTHER CAUSES OF SUPERSENSITIVITY TO ADRENERGIC STIMULI

1. Cocaine

While the phenomenon of supersensitivity in excitable tissues is historically linked to denervation, many procedures can exaggerate responses to adrenergic stimuli. Fröhlich & Loewi (1910) reported that cocaine increased the blood pressure and iris responses to adrenaline. In 1931 Burn & Tainter suggested that cccaine acted like denervation by depressing sympathetic nerve endings. They showed that cocaine could potentiate the excitatory response to adrenaline, but always inhibited the corresponding responses to tyramine. When adrenaline and tyramine were tested on the iris, chronic denervation caused the same changes as did cocaine. Rosenblueth & Cannon (1932) demonstrated potentiation of the nictitating membrane response to adrenaline by cocaine. In 1940 Tripod showed that some other local anaesthetics (stovaine, percaine and butaine) also potentiated smooth muscle responses to adrenaline.

2. Sympathomimetic Amines

Ephedrine was observed by Gaddum & Kwiatkowski (1938) to potentiate the response of rabbit ear blood vessels to adrenaline. Later, Jang (1940) showed that several sympathomimetic amines with low potency could increase the cardiovascular response to adrenaline.

3. Receptor Antagonists

Adrenergic blocking agents in low concentration have also been observed to potentiate adrenaline. Thus Jang (1941) reported enhanced responses to adrenaline in the rabbit ear immediately after low doses of ergotoxin, yohimbine and piperoxan.

In 1958 Nickerson & House reported supersensitivity after chronic administration of phenoxybenzamine. Phenoxybenzamine was given for several days, and the nictitating membrane was supersensitive to noradrenaline when tested two days after the last dose of phenoxybenzamine. The sensitizing effect of the prolonged adrenergic block by phenoxybenzamine appeared to parallel the sensitizing effect of denervation.

4. Drugs Acting on Sympathetic Nerve Endings

Burn & Rand (1957) reported that reserpine decreased the catecholamine content of blood vessels. In 1958athey also showed that treatment with reserpine over a period of 48 hours caused supersensitivity to several catecholamines in perfused vascular beds, isolated artery strips and the nictitating membrane.

Several other drugs which block transmitter release from sympathetic nerves also cause supersensitivity. (For a review see Boura & Green, 1965). These include xylocholine (TM 10) (Exley, 1957), bretylium (Boura & Green, 1959), guanethidine (Maxwell, Plummer, Schneider, Povalski & Daniel, 1960) and bethanidine (Boura & Green, 1963).

A number of pharmacologically different agents may therefore be added to the classical method of denervation as capable of producing supersensitivity in smooth muscle.

D. QUALITATIVE VARIATIONS IN THE TYPE OF SENSITIZATION

Investigation of the causes of supersensitivity is not so much complicated by the procedures which can sensitize smooth muscle as by the fact that their sensitizing effects vary qualitatively. The type of supersensitivity produced by nerve degeneration

depends on the anatomical relation of the nerve to the structure tested, the effects of severing the preganglionic nerve to a structure (decentralization) being different from the effects of division of the postganglionic nerve (denervation).

In 1935 Hampel found that the rate of onset of sensitivity was the same after decentralization and denervation of the nictitating membrane, but that the maximum increase after decentralization was always about half that after denervation. Subsequent denervation of a membrane maximally sensitized by decentralization always markedly increased the sensitivity. Cannon & Rosenblueth (1936) demonstrated that section of the cervical sympathetic trunk resulted in a marked sensitization of the superior cervical ganglion to acetylcholine, as measured by contraction of the nictitating membrane. The response of the membrane to direct stimulation with adrenaline was also potentiated, but not as much as after removal of the ganglion. The normally innervated cat nictitating membrane is more sensitive to adrenaline than to noradrenaline. In 1950 Lockett reported that denervation made the cat nictitating membrane equally sensitive to noradrenaline and adrenaline; in contrast the potency ratio was not changed by decentralization. Innes & Kosterlitz (1954) thoroughly examined the sensitivity of the nictitating membrane after preganglionic and postganglionic denervation. Decentralization caused a general increase in sensitivity to all of a variety of sympathomimetics tested. Changes after denervation, on the other hand, resembled those of decentralization only for the first 24 hours, after which the sensitivity to different sympathomimetics changed. The potency of noradrenaline and cobefrine then increased

to equal that of adrenaline, while responses to tyramine were depressed.

Decentralization and denervation both potentiate many agents as well as adrenaline and noradrenaline. Dale & Richards (1918) reported unspecific increases in the sensitivity of denervated limb vessels to adrenaline, histamine and acetylcholine. Rosenblueth (1932) showed that the denervated nictitating membrane is supersensitive to acetylcholine and histamine. In 1962 Trendelenburg & Weinder demonstrated that the sensitivity to acetylcholine was increased to about the same extent by denervation or decentralization. Trendelenburg (1963a) concluded that decentralization causes a mild supersensitivity which is as prominent with acetylcholine as it is with noradrenaline, and that denervation causes sensitization which is the sum of the sensitization due to decentralization and an additional marked selective sensitization towards catecholamines.

Cocaine produces a supersensitivity which closely resembles that caused by denervation. Thus Innes & Kosterlitz (1954) demonstrated that cocaine changed the responses of chronically decentralized nictitating membrane to resemble that of a chronically denervated one, but had no appreciable effect on the responses of a chronically denervated membrane. Cocaine has also been reported to potentiate cholinergic drugs. For example, Rosenblueth (1932) reported sensitization of the nictitating membrane to acetylcholine. Trendelenburg (1962) found that cocaine potentiated only low doses of acetylcholine in the nictitating membrane, which he interpreted as being

due to the additive effect of noradrenaline released by the cocaine. However, this cannot explain the results of Koppanyi & Feeney (1959) who described cocaine potentiation of acetylcholine on heart rate and vascular responses, where the effects of released catecholamine would be antagonistic. It is generally acknowledged that the potentiating action of cocaine is more marked for catecholamines, and the effect has been compared to the specific component of sensitization to chronic denervation (Innes & Kosterlitz, 1954; Trendelenburg, 1963a).

As well as describing the resemblance between cocaine and denervation effects, Innes & Kosterlitz (1954) also likened potentiation by ephedrine to that of decentralization, since ephedrine also unspecifically enhanced responses to all of a variety of sympathomimetic amines tested. Trendelenburg & Weiner (1962) showed that reserpine also produced an unspecific type of supersensitivity, since acetylcholine was potentiated as much as noradrenaline. However, they concluded that reserpine differs from the other sensitizing pharmacological agents in that several days are required before sensitization is seen. Their inability to demonstrate supersensitivity after short-term treatment with reserpine is difficult to understand, since others, such as Innes (1960) and Brutsaert (1964), observed marked sensitization in several tissues within 24 hours of reserpine administration.

E. THE CAUSE OF SUPERSENSITIVITY: PROPOSED MECHANISMS

1. Effector Changes

a. Removal of inhibition

Dale & Richards (1918) observed enhanced responses of

denervated limb vessels to adrenaline, histamine and acetylcholine. They suggested that the normal tonic effect of nerve impulses restricted the responsiveness to all drugs, and that nerve degeneration permitted an unhampered response to occur. Fleckenstein & Bass (1953) proposed the same concept more explicitly, although their data were restricted to sympathomimetics. Noting that both cocaine and nerve degeneration stop tonic nerve impulses to the muscle cells, they postulated that the sensitivity of the normal membrane is limited by accomodation to the noradrenaline continuously released from the sympathetic nerves.

Burn & Rand (1958b, 1959) and Weiner & Trendelenburg (1962) noted that reserpine treatment and chronic postganglionic denervation both result in loss of the tissue store of noradrenaline. They therefore suggested that continuous slow discharge from the intact store normally keeps sensitivity of the tissue low. Extensive studies in salivary glands have led Emmelin and his colleagues to believe that both sympathetic and parasympathetic nerve endings exert some inhibitory influence on the tissue which normally keeps the sensitivity low. (See Emmelin, 1961, 1965, for reviews). Parasympathetic denervation or prolonged treatment with atropine caused supersensitivity to both cholinergic and adrenergic sialagogues. This supersensitivity was abolished by several days of continuous exposure to any of these stimulants (Emmelin & Muren, 1952). Similarly, sympathetic denervation (Emmelin & Engstrom, 1960) and adrenergic neurone blocking drugs (Emmelin & Ergstrom, 1961) caused a supersensitivity which was not restricted to sympathomimetics.

Thus the concept of the normal state being one of inhibited responsiveness, first proposed by Dale & Richards, is still held by some investigators.

b. Changes in permeability of effector cells

Another early concept which was used to explain supersensitivity was based on changes in permeability. Cannon & Rosenblueth (1936) noted the unspecific supersensitivity in denervated tissues, and suggested that the mechanism was an increased permeability of denervated tissues to stimulating substances. In 1941, Noonan, Fenn & Haege showed that the rate of radioactive potassium accumulation by rat skeletal muscles more than doubled after denervation. These workers included the vasomotor nerves in their motor nerve denervation, so that the greater accumulation of potassium could have been a consequence of changes in vascular tone and permeability. However, Lyman (1942) selectively cut somatic nerves by sectioning the dorsal and ventral roots of the lumbar nerves without damaging the sympathetic nerves. In their experiments early accumulation of radioactive potassium by the gastrocnemius was doubled by denervation, although total uptake was unchanged, which was taken to represent a change in the permeability of the muscle cells. Koppanyi& Feeney (1959) favoured this hypothesis and suggested it as an explanation for their observation of cocaine supersensitization of cardiovascular responses to acetylcholine.

c. Alteration of receptors

In 1937 Clark suggested that cocaine altered the cell receptor "in some manner so that either the rate of association of adrenaline is increased or its rate of dissociation is decreased."

Most investigators since then have ignored the possibility of affinity changes underlying supersensitivity. Maxwell and his co-workers (1958, 1959, 1965) have concluded that the sensitizing action of cocaine and several other agents is on the effector cell. Noting that these agents facilitated the ability of adrenaline and noradrenaline to overcome competitive alpha receptor blockade, these workers suggested that the drugs acted to deform the receptors, thereby increasing their affinity for catecholamines. Maxwell's conclusion has been criticized for being based on inadequate evidence (Trendelenburg, 1963a); however the possibility of this type of change has never been excluded.

2. Changes in Disposal

The foregoing attempts to ascribe a mechanism to supersensitivity all have one feature in common in that they assume that a change occurs in the characteristics of the effector cells, whether it be due to removal of an inhibitory influence, a change in permeability, or a change in the configuration of the receptors. Other attempts to define the cause of supersensitivity also have a common attribute, namely, they propose that altered disposal of the agonist results in an increased effective concentration at the receptors, with the implication that there is no fundamental change in the responsiveness of the effector.

Delayed disposal of agonist would be expected to cause increased duration of responses, and this has often been observed. For example, Meltzer & Meltzer (1903) found that chronic denervation of rabbit ear blood vessels prolonged the constrictor response to

adrenaline. Elliot (1905) noted a similar prolongation in several sympathetically denervated tissues, and inferred that changes in disposal might be related to sensitization. Many other early investigators commented on prolongation of responses in denervated tissues. (For a review, see Cannon & Rosenblueth, 1949). More recently, Innes & Kosterlitz (1954b) have similarly observed that the chronotropic effect of noradrenaline is prolonged after cocaine.

a. Catabolic enzymes

Interest in catabolic enzymes for the sympathetic transmitter was prompted by analogy with the cholinergic nervous system, wherein Brücke (1937) clearly showed that eserine inhibited cholinesterase and thus greatly potentiated the effects of acetylcholine.

In the same year Blaschko, Richter & Schlossmann showed that an amine oxidase present in many mammalian tissues inactivated adrenaline and noradrenaline in vitro. Gaddum & Kwiatkowski in 1938 described ephedrine as an inhibitor of this enzyme and attributed its sensitizing effect to this action. This hypothesis was extended by Philpot in 1940 who demonstrated that cocaine also inhibited amine oxidase in liver slices. Many other reports appeared which indicated a functional role for amine oxidase in terminating the adrenergic response.

In 1946 von Euler provided convincing evidence that noradrenaline was the transmitter at sympathetic nerve endings. The observation that denervation increased the sensitivity of the nictitating membrane to noradrenaline more than to adrenaline (Burn & Hutcheon, 1949; Lockett, 1950) did not reconcile with the earlier

observation of identical rates of destruction for those amines (Blaschkoet al., 1937). However, in 1951 Burn & Robinson showed that noradrenaline was deaminated faster than adrenaline in a mixture of the two. Since this correlated well with their later observations of denervation supersensitivity, Burn (1952) suggested the general theory that supersensitivity to adrenergic stimuli in a result of amine oxidase inhibition.

However, this hypothesis was inconsistent with several facts known at that time. Bacq (1949) indicated that catabolic enzymes other than amine oxidase were important in disposal of catecholamines. Ephedrine (Gaddum & Kwiatkowski, 1938) and cocaine (Jang, 1940) had both been shown to potentiate responses to corbasil, which is not a substrate for amine oxidase (Blaschkoet al., 1937). Thompson & Tickner in 1951 had shown that concentrations of cocaine and ephedrine needed to inhibit amine oxidase in vitro were much greater than those which potentiate adrenaline and noradrenaline in vivo. As early as 1927, Tainter & Chang had shown that cocaine inhibited the action of tyramine which is an excellent substrate for amine oxidase. Philpot (1940) had demonstrated that the effectiveness of several local anaesthetics in causing supersensitivity bore no quantitative relation to their ability to potentiate adrenaline in the nictitating membrane.

In 1953 Foster, Ing & Varagic showed that alpha-cocaine inhibited amine oxidase, but failed to potentiate responses to adrenaline and noradrenaline. Also in 1953, Griesemer and co-workers provided the strongest evidence against the amine oxidase hypothesis,

namely that the potent amine oxidase inhibitor, iproniazid, did not potentiate noradrenaline and adrenaline, but did potentiate tyramine and phenylethylamine, both of which are excellent amine oxidase substrates. It was by then apparent that the proposed role of amine oxidase in supersensitivity to catecholamines was not credible.

In 1936 Bacq reported that phenols such as pyrogallol and catechol caused striking potentiation of adrenaline in several tissues, and attributed this effect to the antioxidant properties of phenols. Axelrod, in 1957, discovered that an O-methylating enzyme, catechol O-methyl transferase (COMT), was important in the normal inactivation of catecholamines. This led Bacq, Gosselin, Dresse, & Renson (1959) to re-investigate the activity of phenols, which he found to be potent in vitro inhibitors of O-methylation of catecholamines. Noting that catecholamine responses were not increased by potent amine oxidase inhibitors, but were increased by COMT inhibitors, he proposed that O-methylation of catecholamines normally limited the responses they cause. In 1960 Wylie, Archer and Arnold confirmed Bacq's observation, but also showed that cocaine did not inhibit COMT. Recently, it has been shown that O-methylation of catecholamines is increased after denervation (Potter et al.,1965). Reduced COMT activity therefore cannot be the cause of supersensitivity after cocaine and denervation.

b. Changes in tissue binding

A variety of techniques have been used to show that sympathetically innervated tissues can take up and bind administered catecholamines. Axelrod, Wiel-Malherbe & Tomchick (1959)

showed a persistent increase in the content of H^3 - adrenaline in several tissues after an infusion or injection. Those tissues with the highest endogenous catecholamine levels showed the greatest increase, heart and spleen being most prominent. Muscholl, (1961) confirmed the marked uptake in cat heart and spleen, finding large increases in their noradrenaline content within 5 minutes after a noradrenaline infusion. Dengler, Spiegel & Titus (1961) showed that slices of cat spleen and heart took up H^3 - noradrenaline by an active process. They calculated the tissue to medium ratio after incubation with varying concentrations of noradrenaline. Tissue accumulation exceeded the medium concentration by 3 or 4 fold at low concentrations, but with increasing concentrations the ratio fell to approach unity, indicating a saturable active transport process which was overshadowed by diffusion at high concentrations.

Nickerson (1965) has discussed the limitations of radioisotope distribution studies, pointing out the possibility that simple appearance of isotope in a tissue may be due to exchange rather than net uptake. Muscholl (1961) and Strömblad & Nickerson (1961) showed by direct measurement that total tissue catecholamine levels were increased after an infusion, which indicated that net uptake had occurred. Iversen (1963) combined both techniques in studying the kinetics of noradrenaline uptake by the isolated perfused rat heart. He showed a net uptake which was initially rapid and was associated with limited exchange of isotope. These appeared to be two different processes of uptake, which had different rate constants, and which were led into separate binding pools within

the tissue.

The sympathetic nerves within the tissues are probably the major site of catecholamine uptake. Hertting & Axelrod (1961) showed that labelled noradrenaline taken up by the perfused cat spleen was liberated by splenic nerve stimulation. No appreciable uptake of injected noradrenaline or adrenaline occurred in denervated rat salivary glands (Strömblad & Nickerson, 1961) or in cat tissues denervated by removal of the superior cervical ganglion (Hertting, Axelrod, Kopin & Whitby, 1961). Iversen, Glowinski & Axelrod (1965) showed that noradrenaline uptake was reduced by 55 to 96 per cent in tissues of immunosympathectomized rats and mice, with the greatest reductions seen in rat spleen and heart.

Many drugs have been shown to interfere with tissue uptake and binding of catecholamines. Whitby, Hertting & Axelrod (1960) showed that uptake of labelled noradrenaline was decreased by cocaine, and that plasma levels were increased. They concluded that this would increase the concentration of active amine at the receptors, and postulated block of catecholamine uptake as the mechanism for cocaine-induced supersensitivity. Hertting, Axelrod & Whitby (1961) distinguished between two groups of drugs which reduced the amount of noradrenaline taken up from an infusion. One group, which included cocaine, was effective only if given before the infusion, while the other group, which included reserpine, reduced accumulation whether given before or after the infusion. The authors emphasized the distinction between uptake and binding, and concluded that some drugs affected only uptake, while others

promoted release, presumably by preventing intraneuronal binding.

Thus the major action of cocaine on the sympathetic nerve ending appears to be only inhibition of uptake. Dengler, Spiegel & Titus (1961) showed that a 10^{-5}M concentration of cocaine inhibited the efflux of catecholamine from previously loaded rabbit heart slices, which indicated that there was no disruption of the intraneuronal binding. Iversen (1963) showed that cocaine block of noradrenaline uptake by the perfused rat heart was dose related, and that a concentration of 10^{-4} M cocaine caused 100 per cent inhibition of uptake.

The actions of reserpine on sympathetic nerve endings are not as clear as those of cocaine. Kopin & Gordon (1962) studied the fate of H^3-noradrenaline released from binding sites by reserpine and tyramine. Tyramine released mainly free noradrenaline, plus a small portion of normetanephrine. In contrast, reserpine released large quantitites of deaminated metabolites and little active catecholamine. The authors concluded that noradrenaline is found in separate pools within tissues and that drugs may have selective actions on these pools. Histochemical studies on rat iris by Hillarp & Malmfors (1964) showed that reserpine did not prevent the accumulation of noradrenaline by sympathetic nerves, but that retention was brief. Kopin, Hertting & Gordon (1962) reported that depletion of noradrenaline by reserpine was not associated with inhibition of uptake. Hearts taken from reserpine treated rats and perfused with H^3-noradrenaline (0.1 micrograms/min.) accumulated large amounts of noradrenaline, but retention was brief compared to normal hearts.

However, because such a large amount of noradrenaline was given, the significance of these results in terms of normal uptake process is questionable; such a large concentration gradient would be expected to promote considerable uptake by simple diffusion. Hillarp & Malmfors (1964) reported histochemical data which seem to be more meaningful. Using fluorescent microscopy they showed transient uptake of noradrenaline by neurons in the irides of reserpine treated rats.

Furchgott,Kirpekar, Rieker & Schwab (1963) described physiological experiments from which they deduced the existence of specific sites in the neuronal membrane with which noradrenaline must combine before being transferred to sites of storage or inactivation within the nerve. Cocaine and reserpine both markedly decreased the responses to tyramine by isolated rabbit aorta and by guinea pig and cat left atria. Cocaine still potentiated noradrenaline after reserpine, and tyramine, which now caused no response itself, also potentiated noradrenaline. Incubation with noradrenaline temporarily restored the response to tyramine in tissues which had been depleted of noradrenaline by reserpine. However, restoration was prevented if either cocaine or tyramine were in the tissue during incubation. If the sympathetic nerve endings in cat atria were removed by prior denervation, incubation with noradrenaline caused no change in the response to tyramine.

Competition of cocaine, tyramine and noradrenaline for a common transfer site in the nerve membrane was suggested to explain these data. Reserpine could reach the storage sites

directly, and did not affect the transfer mechanism. Block of the transfer mechanism by cocaine or tyramine, however, prevented subsequent refilling of the storage sites, indicating the essential nature of the transfer mechanism in uptake. Cocaine would therefore potentiate noradrenaline by preventing its removal from the vicinity of receptors, and would inhibit tyramine by blocking its access to noradrenaline storage sites on which it normally acted. Because replenishment of reserpine-depleted stores was known to reach only a fraction of the normal levels (Hertting, Axelrod, & Whitby, 1961), yet large responses to tyramine were obtained, separate storage sites were postulated, one of which contained major quantities of noradrenaline, but was not accessible for physiological release, and a second smaller available store which was the main site of tyramine action, and which released most of its contents in the immediate vicinity of receptors.

The uptake hypothesis of supersensitivity is embodied in these concepts. When data from Axelrod's laboratory showed that cocaine (Whitby et al., 1960) and denervation (Hertting, Axelrod, Kopin, & Whitby, 1961) decreased the accumulation of labelled noradrenaline, the authors proposed a causal relation between this and the associated supersensitivity. Considerable data has been accumulated demonstrating associations between changes in uptake and supersensitivity. Drugs other than cocaine which cause supersensitivity, such as bretylium and guanethidine, were shown to inhibit noradrenaline uptake (Hertting, Axelrod & Patrick, 1962). Iversen (1965a) found that the affinity of the

uptake mechanism for noradrenaline was greater than for adrenaline, which correlates with the greater potentiation of the former by cocaine and denervation. Trendelenburg (1966) showed that the loss of catecholamine after the removal of the superior cervical ganglion required about 16 hours, coinciding with the time of onset of supersensitivity. (For detailed reviews of data relating sensitivity to uptake, see Trendelenburg, 1963a, 1966; Axelrod, 1965; Costa, Boullin, Hammer, Vogel & Brodie, 1966).

Many reports have provided evidence that the interactions of sympathomimetics and cocaine with the uptake mechanism are competitive. Muscholl (1961) showed that increasing the concentration of noradrenaline in an infusion made it necessary to use larger doses of cocaine to inhibit uptake by cat heart and spleen. Trendelenburg (1961) showed that increasing doses of cocaine caused a graded parallel shift to the right of dose-response curves to tyramine on cat nictitating membrane, heart rate and blood pressure, in a manner typical of a surmountable antagonist. Lockett & Eakins (1960) showed the noradrenaline releasing action of tyramine was blocked by cocaine; increasing the dose of tyramine could overcome the inhibition.

Iversen (1965b) showed that a large number of sympathomimetic amines compete with noradrenaline for the uptake process in the isolated rat heart. The effectiveness with which they competed varied, and was taken as a measure of their affinity for the uptake mechanism. The inhibition could be separated into two processes, one occurring at low concentrations ($uptake_1$), another

at higher concentrations ($uptake_2$), both processes following classical Michaelis-Menten kinetics. The amines could be classified into 2 separate groups according to their relative affinities for each process. It is clear from the results of these and many other experiments that uptake occurs by specific active processes for which competition occurs among the compounds which are taken up. Drugs, such as cocaine, which combine with the uptake sites but are not transferred to storage sites, would block the uptake of amines which are normally taken up. According to the uptake hypothesis, then, the resulting increase in concentration of "free" amine would be seen as an increase in response to its direct actions on the receptors.

F. THE CURRENT STATUS: INCONSISTENCIES

The extensive investigations into supersensitivity probably enable two statements which would not be disputed: (1) Certain sensitizing procedures cause increased sensitivity to many different agonists. (2) Other procedures cause a supersensitivity which is especially marked for the response to noradrenaline.

The variations on the theme that the normal presence of mediator maintains a low sensitivity might explain the unspecific supersensitivity, but it is difficult to see how the disproportionate sensitization of noradrenaline can be thus explained. When the nerves are cut, the accommodation-producing impulses (Fleckenstein & Bass, 1953) presumably cease immediately, yet supersensitivity does not begin until about 24 hours and takes several days to reach maximum. On the other hand, cocaine increases sensitivity as soon

as it is given. Obviously, the removal of accommodating influences cannot fully explain the complexities of supersensitivity.

The uptake theory of supersensitivity has been referred to as explaining the specific sensitization to noradrenaline (Trendelenburg & Weiner, 1962; Kirpekar, Cervoni & Furchgott, 1963). Not only is it irrelevant to unspecific supersensitivity, but several inconsistencies make it an unlikely explanation for specific sensitization to noradrenaline.

According to the uptake theory, reduced uptake of catecholamines enables a greater concentration to reach the site of action. Sensitization should therefore be independent of the nature of the receptor involved. Data with regard to response mediated by beta receptor stimulation are conflicting. Burn & Tainter (1931) showed that cocaine did not enhance, but inhibited the response to stimulation of beta receptors in the virgin cat uterus and cat atrium. Burrett (1940) found that the maximum increase in heart rate after noradrenaline was increased by sympathetic denervation, and Peralta & Lizaralde (1946) reported that the duration of the chronotropic response to adrenaline was increased. Innes & Kosterlitz (1950) showed an increase in the chronotropic response to noradrenaline in cat hearts after cocaine and denervation, but were unable to find any change in the response to adrenaline. Stafford (1963) reported that cocaine, guanethidine and phenoxybenzamine all increased the chronotropic and inotropic changes to noradrenaline and adrenaline in isolated rabbit atria, but had no effect on the responses of isolated rabbit duodenum or rat uterus.

The sites of major catecholamine uptake which are removed by cocaine are in the nerve endings (Hertting, Axelrod, Kopin & Whitby, 1961; Potter, Cooper, Willman & Wolfe, 1965). Yet it has been possible to demonstrate supersensitivity to catecholamines caused by cocaine in the isolated human placenta, a nerve-free preparation (von Euler, 1938).

Since the uptake sites which cocaine may block must be present to satisfy the uptake theory, observations on the effect of cocaine on tissues with impaired or blocked uptake are pertinent. Furchgott et al. (1963) showed that cocaine retained its full potentiating effect even when the catecholamine binding mechanism had been drastically altered by reserpine. Similarly, when the sympathetic nerves to the nictitating membrane had been cut, and sufficient time allowed for degeneration to occur but not for supersensitivity to be maximum, cocaine still had full sensitizing potential (Innes, unpublished observations).

Changes in distribution of agonist within the tissue, such as occur when uptake is altered, will have an insignificant effect on the amount of drug in the bathing fluid. Since the concentration of drug in the bathing fluid is in equilibrium with that in the tissue, the amount available for combination with receptors will not change. In the whole animal, where uptake sites in all tissues will remove agonist from the circulation, the final concentration in equilibrium with receptors could be significantly affected. Yet, many observations of supersensitivity are reported on isolated preparations. In view of this argument, these observations must find another explanation.

Maxwell, Wastila & Eckhardt (1966), who also noted the argument cited above, have recently reported that uptake and sensitivity can be functionally separated in the rabbit aorta strip. Cocaine, guanethidine and methylphenidate all caused a concentration dependent reduction in the uptake of H^3-noradrenaline from the bathing fluid. Until the reduction in uptake reached 30-70 per cent, the response increased as the uptake became less. However, further increases in the concentration of potentiating drug caused considerable increases in response with no additional decrease in uptake. Maxwell has postulated that sensitizing drugs such as cocaine act directly on the alpha receptors to deform them.

A striking point against the uptake hypothesis of supersensitivity is that denervation supersensitivity can be prevented by chronic treatment with adrenaline. Karr & Nickerson (unpublished observations) unilaterally denervated cat submaxillary glands by removing the superior cervical ganglion. To one group, depots of adrenaline in oil were given during the period of nerve degeneration. After 14 to 30 days, treatment was stopped and both salivary glands were tested in an acute experiment 18 to 24 hours later for sensitivity to noradrenaline and for uptake after a large dose of noradrenaline. The other group received no treatment after operation and were similarly tested in acute experiments. Noradrenaline uptake was significantly reduced in all denervated glands; sensitivity to noradrenaline was increased in the denervated glands of the untreated group, but the denervated glands in the group given adrenaline in oil showed no greater sensitivity then their innervated

controls. These experiments showed that catecholamine uptake could be reduced without the occurrance of supersensitivity.

G. STATEMENT OF THE PROBLEM

The events between addition of a drug to a tissue and the measured response are many and complex. In previous investigations of supersensitivity, many events seem to have been ignored whch might well relate to the problem. We therefore wanted to break the stimulus-response sequence down into fundamental components in order to visualize possible points of attack more easily. In Figure 1 we present a scheme showing arbitrary divisions within the events leading to a response. The purpose of these divisions is simply to facilitate consideration and discussion of the problem; they are not intended to represent any intrinsic change or advance in our understanding of the events which normally lead to a response.

If mass action kinetics prevail, the combination of a drug with receptors to produce a response can be simply presented in reaction form:

$$D + R \underset{k_2}{\overset{k_1}{\rightleftharpoons}} DR \longrightarrow \text{ACTIVATION}$$

D is drug concentration, R is the receptor pool, and k_1 and k_2 are the rate constants for association and dissociation of the drug-receptor complex, DR. Thus k_2/k_1 is the dissociation constant for the complex, and its reciprocal, k_1/k_2 is sometimes known as the affinity constant. Where D is an agonist, the complex DR leads to a response which is related in some way to the size of DR.

In the figure, this equation has been expanded to include, in general terms, the operation of the adrenergic mechanism. When

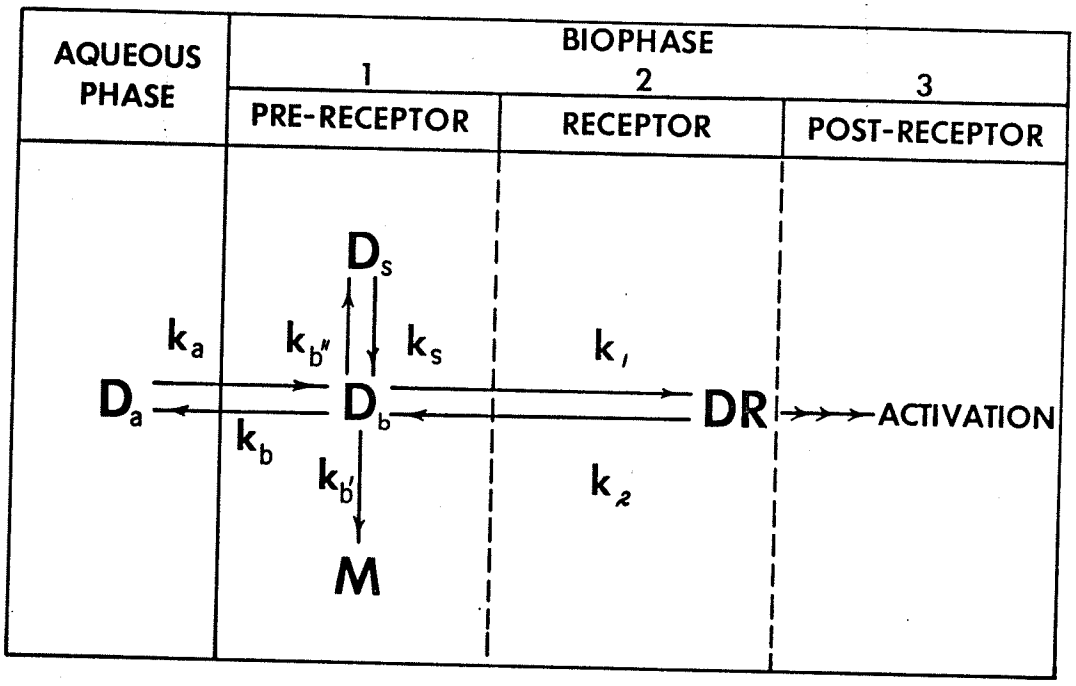

Fig. 1 A SCHEME FOR THE DOSE-RESPONSE SEQUENCE

A simplified scheme to represent events influencing the response of a tissue to an agonist, with particular reference to a catecholamine. D_a and D_b are concentrations of the agonist in the aqueous phase and biophase respectively. Rate constants k_a and k_b govern passage of drug between phases. Within the biophase, the rate constant k_b' represents the rate net loss due to enzymic destruction, and the rate constants k_b'' and k_s govern the amount of drug entering and leaving the bound form (D_s) in storage sites within the biophase. Rate constants k_1 and k_2 represent the association and dissociation constants for combination of drug from D_b with receptor sites (R).

a drug such as noradrenaline is added to the fluid bathing an isolated tissue containing alpha receptors, the bathing fluid to which the drug is added (aqueous phase) is separated from its site of action by an ill-defined barrier which creates a second phase known as the biophase (Fergusson, 1939). Events within the biophase may be divided into the three categories illustrated in the figure.

1. Pre-receptor

When a concentration of drug (D_a) is added to the aqueous phase, pre-receptor events will determine both the concentration of drug (D_b) which reaches equilibrium with its site of action in the biophase, and the rate at which equilibrium is achieved.

(a) D_b will always move toward equilibrium with D_a. Changes in permeability of the biophase barrier, or in related transport mechanisms may affect both the rate at which equilibrium is reached when the drug is first added, and the rate of reduction in D_b when D_a is removed.

(b) Enzymes within the biophase will destroy the drug. These reactions will reach their own equilibria (not shown), but because the drug is a substrate for these enzymes, D_b will be reduced at an overall net rate represented in the figure by the rate constant k_b'. As D_b falls, more drug will move from D_a into the biophase. Eventually the concentration of D_a may fall significantly, which will be reflected in the lowering of D_b and reduction of the response. If the enzymatic reactions are sufficiently rapid, and the attainment of equilibrium between D_a and D_b is normally slow, then these reactions may influence the concentra-

tion which reaches equilibrium in an isolated tissue.

(c) The storage mechanisms within the biophase will take up a certain concentration of drug, D_s. The rate at which equilibrium between D_a and D_b occurs when drug is first added will be influenced in part by this process. If the maximum possible value for D_s is large enough relative to D_a, as may be the case in the whole animal where the drug is added to the circulation, then the final value of D_b may also be influenced. However, when the volume containing D_a is large relative to the tissue volume, then D_a represents a reservoir of drug which will not be significantly altered by the relatively small amount drawn off as D_s. If D_a remains unchanged by the removal of D_s then the free concentration in the biophase, D_b, will not be altered. Unlike the enzymatic removal of drug, storage does not in itself lead to destruction. Thus at equilibrium the amount of drug being bound ($D_b \cdot k_b''$) will be balanced by an equal amount dissociating from the storage mechanism ($D_s \cdot k_s$) and there will be no continued drain on D_b.

2. Receptor

D_b is the "active" concentration of drug which is free to combine with the receptors. Since a reversible equilibrium develops between drug and receptors, the formation of the complex DR will be governed by:

(a) Drug concentration (D_b)

(b) Receptor "concentration", or the size of the receptor pool, R.

(c) The rate constant, k_1, for association of the drug and receptors, and the rate constant k_2 for dissociation of the complex DR. The term "affinity" is usually used to describe the readiness with which the receptors form a complex with drug. The ratio of the rate constants k_1/k_2 is called the affinity constant (K_a) and is a measure of this property.

3. Post-receptor

Each activated receptor will initiate a series of events which lead to a final response, such as secretion or shortening of actomyosin filaments. It is now believed that the quantum of stimulation provided by each activated receptor is not fixed, but varies with the properties of the drug. The terms intrinsic activity (Ariëns, 1954) and efficacy (Stephenson, 1956) have been used to describe this property which, together with affinity, determines the activity of a drug is a given tissue. If response were directly proportional to the number of receptors occupied, then the maximum response obtained with any drug would be a function of its intrinsic activity. However, we now realize, through the work of Stephenson (1956) and Nickerson (1956) that there is a ceiling for the maximum response to some drugs with high efficacy which is limited by events after the receptors rather than the number of receptors. A drug capable of causing such a "full" maximum response when all the receptors are occupied is called a full agonist. Agonists with efficacy less than that necessary to give the full maximum response when all the receptors are occupied are called partial agonists (Stephenson, 1956), and

represent an intermediate class between full agonists and antagonists, which have no efficacy. Partial agonists will cause a response which is proportional to the amount of drug-receptor complex, and maximum response, representing occupation of all receptors, will be a measure of relative efficacy. On the other hand, increasing the efficacy beyond that necessary to give a full maximum response when all receptors are occupied will enable this response to be elicited when a fraction of the receptors are occupied. Stephenson (1956) has pointed out that there will be spare receptors for such an agonist, so that a portion of the total receptor pool could be eliminated by irreversible blockade without reducing the maximum response attainable. Another consequence of this concept is that the response to a drug for which there are spare receptors will not be proportional to receptor occupancy throughout the whole dose range.

The early investigators of supersensitivity speculated on its cause in very broad terms, probably because of their ignorance of the details of the normal response. Most of our progress is understanding the adrenergic mechanism has been limited to the processes of uptake, binding and metabolism which are important mainly in terms of the concentration of the drug in equilibrium with the receptors, the pre-receptor events of Figure 1. In view of the direction that research on the adrenergic mechanism has taken, it is not surprising that the approach to the question of supersensitivity in smooth muscle has followed the same path. Thus both the currently popular uptake hypothesis, and the amine

oxidase theory before it, emphasized altered disposal of the agonist as causing supersensitivity by increasing the concentration in equilibrium with the receptors.

This approach does represent a valid possibility in attempting to explain supersensitivity; however other areas of investigation included in the receptor and post-receptor categories have been neglected and deserve investigation. Two general possibilities emerge:

1. Changes in the chemical or physical properties of the receptor could result in either an increased binding capacity of the receptors for agonist (increased affinity) or an increased quantum of stimulation per activated receptor (increased efficacy). Receptor antagonists, which are not metabolized rapidly and which do not activate post-receptor processes, would be useful in distinguishing between these characteristics.

2. Change in post-receptor events could link up more receptors to muscle fibres, thereby increasing the number of effective receptors. An assumption required for this possibility is the existence of muscle fibres which are not normally activated when the tissue is fully contracted by stimulating the receptors in question.

The purpose of the experiments reported in this thesis was to examine in one tissue the possible influence of pre-receptor, receptor, and post-receptor events in supersensitivity to catecholamines. In obtaining such a general view of the contraction process relative to supersensitivity, we have not been able to examine

any one area thoroughly. As an inevitable consequence, the results often suggest other experiments which will be discussed in the thesis.

H. SELECTION OF THE EXPERIMENTAL OBJECT

All the experiments reported have been done on the isolated cat spleen. Although experiments in the whole animal offer the advantage of being less artificial, they have certain inherent disadvantages when applied to a study of supersensitivity: (a) the question of drug concentration at the site of action, always an unknown, is especially unpredictable in the whole animal; (b) when drugs which cause a response are added, actions remote from the tissue whose responses are being studied, including reflex effects, cannot be completely eliminated.

For the particular problem at hand, certain basic attributes are required of the tissue chosen for the isolated preparation:

(a) It should respond to adrenergic stimuli.

(b) It should have rich sympathetic innervation, and stimulation of these nerves in vivo should be known to cause a significant response.

(c) It should be possible to cut the post-ganglionic sympathetic nerves to produce chronic sympathetic denervation.

(d) It should respond to agonists other then sympathomimetics.

(e) It should show supersensitivity to adrenergic stimuli both in vivo and as an isolated preparation after procedures which normally cause supersensitivity in smooth muscle.

The isolated cat spleen has all these attributes. However, it has the additional properties of having more than one type of smooth muscle, as well as possessing a considerable mass of reticuloendothelial tissue which lacks smooth muscle elements. The presence of non-muscular tissue seems to be a feature of most smooth muscle preparations currently available for in vitro studies. The possibility that these non-muscular elements may appreciably influence the distribution of drugs has never been ruled out, although recent histochemical studies (e.g. Malmfors, 1965) indicate this is not the case for the catecholamines at least.

METHODS

A. PREPARATION OF THE ISOLATED SPLEEN STRIP

Cats or kittens, 0.5 to 4 kg, of either sex were killed by a blow on the head. The spleen was removed and placed in Krebs-Henseleit solution at 4°C as quickly as possible. The dissection was done on a piece of filter paper laid on an inverted Petri dish and saturated with the bathing solution. Strips 20 mm long and about 2 mm wide were cut from the edge of the spleen so that all strips would have a similar content of capsular and vascular tissue. Stretching the tissue was carefully avoided during all phases of handling and cutting. Each strip was attached by a loop of terylene thread to a glass hook which was then inserted in an organ bath. The strip was suspended vertically, and attached by a terylene thread to a light Palmer frontal writing lever.

The organ bath contained about 10 ml of bathing fluid kept at 38 ± 0.5°C, and was drained and filled through openings at the bottom of the chamber. After suspending the strips, an equilibration period of one hour was allowed before starting an experiment. During this time, and throughout the experiment when drugs were not being added to or removed from the bath, the bathing fluid was routinely replaced at 10-15 minute intervals.

Isotonic contractions against 1 g tension were recorded on a kymograph at 8 times magnification and 1 mm/min paper speed.

B. BATHING MEDIA

The standard bathing fluid was Krebs-Henseleit solution of the following composition: NaCl 118, KCl 4.7, $CaCl_2$ 2.4, KH_2PO_4 1.1, $MgSO_4$ 1.2, $NaHCO_3$ 25, and glucose 11 mM. The solution was saturated

with a mixture of 95% O_2 and 5% CO_2 before the experiment, and this mixture was also bubbled through the fluid in each organ bath throughout the experiment.

Media for calcium depletion

In experiments where the tissues were to be bathed in a medium without calcium, a calcium-free solution was prepared by omitting $CaCl_2$ from the standard Krebs-Henseleit formula. When the depletion of tissue calcium was being promoted, the chelating agent disodium ethylenediaminetetra-acetic acid (EDTA 0.03 mM) was added. We have adopted the convention of Hinke (1965), who distinguished this solution from the calcium-free one by calling it a zero-calcium solution.

C. MEDIUM FOR SUSTAINED RESPONSE TO CATECHOLAMINES

Prolonged contractions to high concentrations of catecholamines were required in some experiments. Trace quantities of divalent ions normally present in the bathing media promote the rapid oxidation of catecholamines. These were removed by adding EDTA (0.03 mM) to the standard Krebs-Henseleit solution as suggested by Furchgott (1955). In this concentration, no significant reduction in the relatively high calcium or magnesium concentrations would be expected.

D. DRUGS

Agonists

Stock solutions containing 10 mg/ml were made of all agonists. The bitartrates of noradrenaline and adrenaline, and the hydrochlorides of tyramine and isoproterenol, were prepared in 0.1 N HCl.

Acetylcholine chloride and histamine acid phosphate were dissolved in distilled water.

Antagonists

Phenoxybenzamine hydrochloride (Dibenzyline), 10 mg/ml, was dissolved in 25 ml propylene glycol and acidified with a few drops of 5 N HCl. Phentolamine HCl (Regitine, Ciba) and pronethalol HCl (Alderlin, Ayerst) were made in a stock solution containing 1 mg/ml of distilled water.

Other Drugs

A stock solution of cocaine HCl, 1 mg/ml, was made in distilled water. A reserpine stock solution of 5 mg/ml was prepared by dissolving 100 mg in 2.0 ml glacial acetic acid, 2.5 ml propylene glycol, and 2.5 ml 95% ethanol and adding sufficient water to make 20 ml.

All stock solutions were stored at 4°C. Dilutions were made in 0.9% NaCl on the day of an experiment, and refrigerated when not in use. Dilutions of catecholamines were acidified by adding HCl, 0.01N. The dilutions were such that the bath volume was rarely increased by more than 0.5 ml. Additions were made with a 1 ml tuberculin syringe or blow-out micropipettes with capacities of from 0.01 to 0.5 ml. A solution of KCl, 1.8 M, in distilled water, was used to test the response to potassium in some experiments.

E. TREATMENT OF CATS WITH RESERPINE

Cats to be depleted of noradrenaline stores were given reserpine 1.0 mg/kg intraperitoneally 16-24 hours before an experiment. It has been clearly shown that depletion of tissue noradrenaline

results in loss of responses to moderate doses of tyramine (Carlsson, Rosengren, Bertler & Nilsson, 1957; Burn & Rand, 1958a; and many others). Spleen strips were tested for depletion by adding 10^{-5} g/ml tyramine to the bath at the beginning of the experiment.

F. DENERVATION OF THE CAT SPLEEN

Postganglionic denervation of the spleen was done by dividing the splenic nerves. Anesthesia was induced with pentobarbital (25 mg/kg) and maintained when necessary with open drop ether. A midline abdominal incision exposed the spleen which was held in sterile saline-soaked gauze so that the hilar surface presented. The connective tissues surrounding the vessels close to the hilum were carefully teased away with a fine forceps. The exposed vessels were examined closely and any non-vascular tissue in the adventitia was removed. This procedure was done only on the half of the spleen which lies dorso-medially in the anatomical position. This half always had less dense vascular branching near the hilum, and the tissue it provided was adequate for an experiment. The denervated and innervated parts of the spleen were divided between two ties of a thick string. The spleen was replaced, the wound sutured, and 0.5 ml of Fortimycin-$\frac{1}{2}$ (Ayerst), containing 100,000 I.U penicillin G procaine and 0.125 g streptomycin, given intramuscularly.

The entire procedure was done aseptically with sterile instruments. The operation usually took about 30 minutes after the induction of anesthesia, and the cats were allowed to recover in a heated cage.

G. EXPERIMENTAL PROCEDURES

1. Dose-response Curves

Dose-response curves for spleen strips were obtained by testing graded increases in the $\log_{10}$ concentration of agonist. Low doses were always tested first, and the initial test dose was selected to be slightly below or above the threshold for a response. Subsequent doses were spaced at approximately half or, in some experiments, one third log intervals. When maximum contractions were being tested, the dose increase was continued until no further increase in response occurred. In most types of experiments, the tissues were allowed to recover from each contraction before the next dose was added. In longer experiments, and where each strip was its own control, cumulative additions of agonist were tested. Each addition then was made after a plateau response to the previous dose had been reached. The concentration added each time was the difference between the existing bath concentration and the desired concentration. Inactivation of drug in the bathing fluid was ignored.

2. Protection Against Cocaine-induced Supersensitivity

Several drugs were tested for their ability to prevent the potentiating effect of cocaine on catecholamines. The design of these experiments is summarized in Table 1. Four strips from the same spleen were used in each experiment. First, reproducible control responses to adrenaline or noradrenaline (10^{-7} to 3×10^{-6} g/ml) were obtained in each strip. Then a high concentration of protecting drug was added to two tissues. This was 10^{-5} or 5×10^{-5} g/ml for all drugs except phentolamine, which was 3×10^{-6} g/ml.

TABLE I

DESIGN FOR EXPERIMENTS ON PROTECTION AGAINST COCAINE-INDUCED SUPERSENSITIVITY

Four strips are required for each experiment. Small crosses (x) denote addition of a small dose of noradrenaline or adrenaline. Large crosses (X) denote addition of protecting drug or cocaine. Strip 1: time control. Strip 2: cocaine. Strip 3: protecting drug + cocaine. Strip 4: control for effect of protecting drug.

PROCEDURE	STRIP 1	2	3	4
1. Test Response	x	x	x	x
2. Add Protecting Drug wait 5 minutes	-	-	X	X
3. Add Cocaine wait 5 minutes remove drugs	-	X	X	-
4. Test Response	x	x	x	x

Five minutes later, cocaine 10^{-5} g/ml was added to one protected strip and one other strip, during which time the protecting drug remained in the baths. After another 5 minutes both drugs were removed and the tissues washed at 5 minute intervals for 30 minutes. Responses to catecholamine were then tested regularly until the response of the strip which received only the protecting drug returned to its original value. At this time the responses in each of the four strips were expressed as a percentage of the control values in the same strip. The effect of the protecting drug on cocaine potentiation could then be determined, and was properly controlled for the effects of time and protecting drug on the response to catecholamine.

3. Determination of pA_{10}

The method of Schild (1947) was modified to determine pA_{10} values for phentolamine against noradrenaline in normal and super-sensitive spleen strips. pA_{10} is the negative logarithm of the concentration of an antagonist which will reduce the response of a ten fold increase in agonist concentration to the response caused by a unit dose of the agonist. A unit dose of noradrenaline was tested which would cause a significant response in the lower region of the dose-response curve. A dose of phentolamine was added to the bath. Five minutes later the response to a tenfold dose of noradrenaline in the presence of the phentolamine was determined. After return to baseline, the unit dose of noradrenaline was repeatedly tested until the phentolamine was removed, as indicated by return of the response to near its control value and by failure to increase further. A second dose of phentolamine was added to

the bath and the same procedure of testing a tenfold dose repeated. Doses of phentolamine were selected which would result in responses slightly above and slightly below the response of the unit dose. In many experiments the response to the unit dose varied in either direction by about 10 per cent. When this occurred, the response to the tenfold dose of noradrenaline in the presence of phentolamine was always compared with the immediately preceding unit dose. The ratio of the differences above and below the unit responses was used to interpolate between the log doses of phentolamine used. In this way, the logarithm of the dose of phentolamine which would cause the response to a tenfold dose to equal that of a unit dose was calculated.

4. Dose-ratio Experiments

The dose-ratio (Gaddum, Hameed, Hatheway & Stephens 1955) was used to estimate the antagonism of noradrenaline by phenoxybenzamine. Dose-ratio is most commonly used to indicate the amount of antagonism by a competitive antagonist, where dose-response curves with and without antagonist are parallel throughout their whole length. Phenoxybenzamine is a non-equilibrium antagonist, and the dose which had to be used reduced the maximum response to noradrenaline, so that the dose-response curves were not parallel throughout. The method suggested by Schild (1949) and Gaddum (1957) for measuring non-competitive antagonism, where maximum responses are also reduced, was applied to phenoxybenzamine. Thus, the dose-ratio was always calculated at a response level equal to 50 per cent of the reduced maximum response, as shown in Figure 2. The dose-

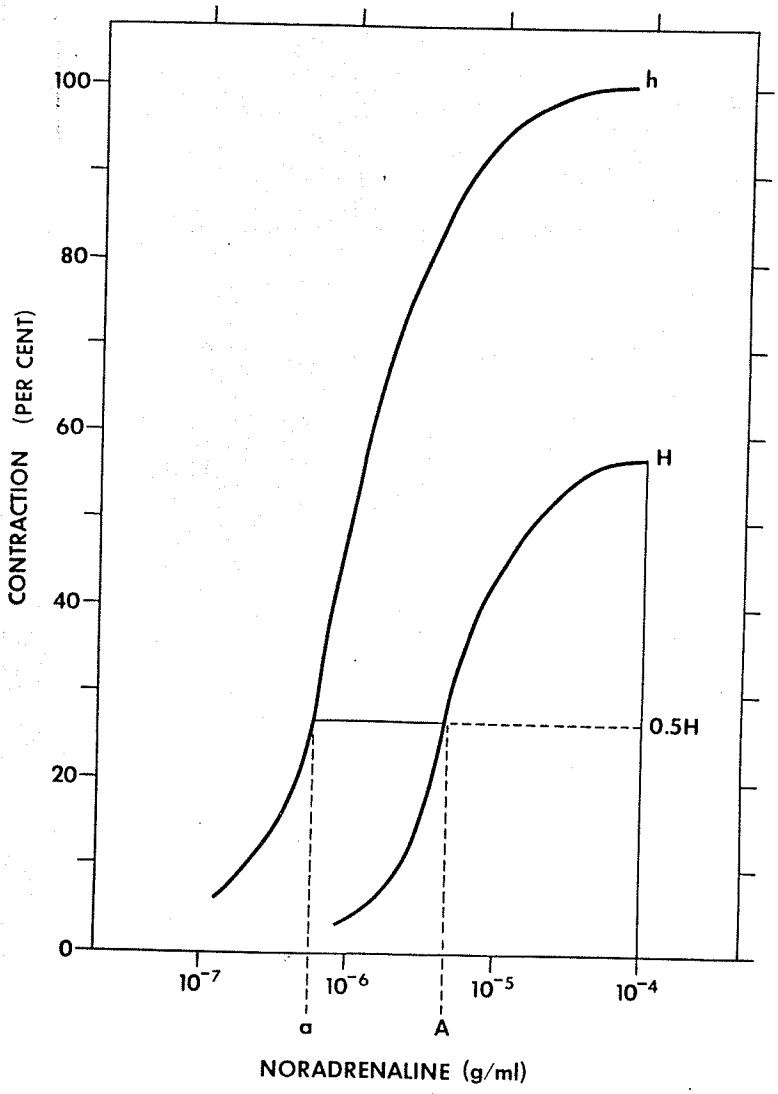

Fig. 2 CALCULATION OF PHENOXYBENZAMINE DOSE-RATIOS.

Schematic curves illustrating the method of calculating the dose-ratio for phenoxybenzamine. Dose-response curves for noradrenaline are obtained before (maximum contraction h) and after (maximum contraction H) phenoxybenzamine. The doses of noradrenaline (a and A) which cause a contraction equal to 0.5H are measured from the curves, and the dose-ratio is calculated as A/a.

response curves with and without antagonist never noticeably deviated from parallel in this region with the dose of phenoxybenzamine used.

A full dose-response curve to noradrenaline was obtained by addition of cumulative doses. After 2 hours, phenoxybenzamine 2.5 x 10^{-8} or 5 x 10^{-8} g/ml was applied for five minutes and removed. Thirty minutes later, after several washings, the cumulative dose-response curve to noradrenaline was determined again. The maximum response to noradrenaline after phenoxybenzamine was invariably less than the corresponding control maximum. The response equal to 50 per cent of the reduced maximum was measured, and the dose-ratio at this specified response was calculated by dividing the dose producing this response after phenoxybenzamine by the dose producing it originally in the same strip.

5. Test for a Partial Agonist

A modification of the method proposed by Ariens, van Rossum & Simonis (1956) was used to test isoproterenol for partial agonist activity. Full cumulative dose-response curves to isoproterenol were obtained in four strips of spleen from the same cat. After a recovery period of 2 hours, during which the tissues were washed at 15 minute intervals, different doses of noradrenaline were added to each bath. These were chosen to give a range of responses above and below the isoproterenol maximum. When these contractions reached their peak, isoproterenol was tested in each strip as before, with the noradrenaline still present. All responses were calculated as a percentage of the original isoproterenol maximum, and the resulting curves were plotted for comparison.

6. Steady-state Contractions

Spleen strip contractions due to noradrenaline or isoproterenol do not fall off immediately after the highest point is reached if EDTA is in the bathing fluid. A steady level of contraction lasts for up to 1 hour, and then the contraction undergoes only a slow linear decline. This is referred to as a steady-state contraction. A dose of 5×10^{-5} or 10^{-4} g/ml of noradrenaline causes a maximum contraction which is sustained for 2 hours or more, probably due to the excess of drug used.

In experiments where this type of maximum response was studied, sufficient time was allowed to confirm that a steady-state obtained after the peak of contraction was reached. Two types of experiment were then done. In one, cocaine was added and any change from the steady-state was measured. In another, phenoxybenzamine was added and the rate of decay of the response was determined as shown in Figure 3. The difference in height between the original and the reduced maximum levels was taken as 100 per cent, and the values for 25, 50 and 75 per cent were measured. The times required to reach these points from the time of addition of phenoxybenzamine were then measured and thus provided an expression of the rate of decay.

7. Response to Restoration of Calcium

For studies on the effect of varying the calcium ion concentration, spleen strips were first bathed in zero-calcium medium to remove as much tissue calcium as possible. During this time they were stimulated with increasing concentrations of histamine and either adrenaline or noradrenaline to remove bound calcium until

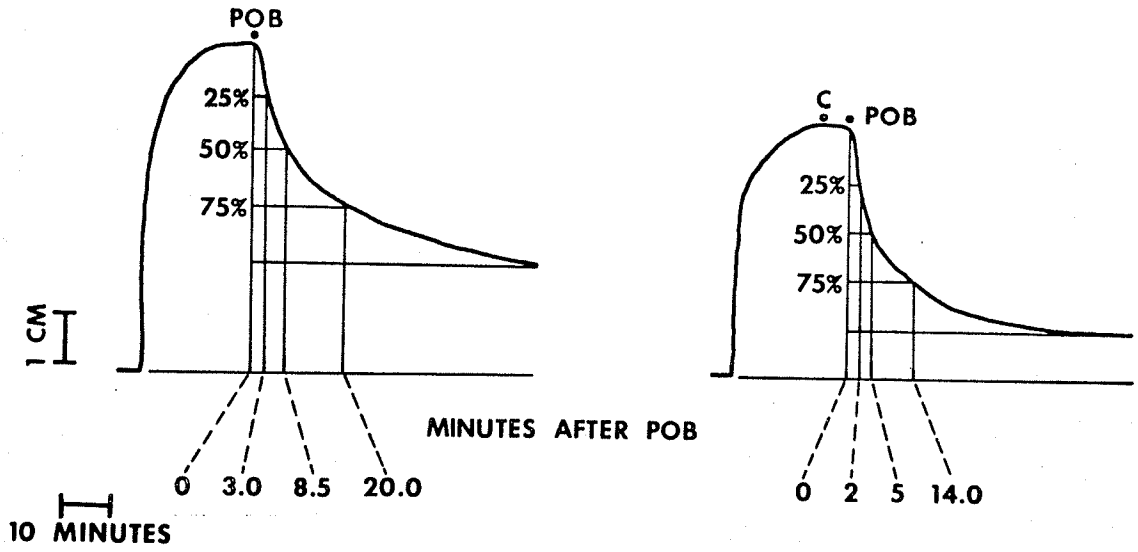

Fig. 3 CALCULATION OF DECAY RATE OF STEADY-STATE CONTRACTION AFTER PHENOXYBENZAMINE.

Tracings of experimental steady-state contraction to noradrenaline (10^{-4} g/ml) showing the method of calculating the decay rate after adding phenoxybenzamine (POB, 10^{-6} g/ml). The asymptote for decay is estimated and assigned the value 100%. Lines projected as shown from this point and from the point of addition of phenoxybenzamine are used to calculate the values for 25, 50 and 75% decay and their corresponding times. The effect of cocaine (10^{-6} g/ml), added at C, is described in the results.

there was no more reduction of the response to repeated administration of the same dose. The zero-calcium medium was then replaced by a calcium-free one. Noradrenaline (10^{-4} g/ml) was added and left in the bath for the rest of the experiment. This dose caused a small, but sustained contraction which could be increased by addition of calcium. When this initial response reached a steady-state, calcium was restored to the bathing fluid in small increments. Each addition was made only when the contraction due to the preceding one had stopped increasing, so that a cumulative dose-response curve to calcium restoration was obtained. The initial response of calcium-depleted strips was from 5 to 22 per cent of the maximum contraction after addition of calcium. The magnitude of the response to each addition of calcium was calculated as a percentage of the total maximum after subtracting the initial response to noradrenaline. These responses are plotted against the calcium concentration in the bath expressed as a percentage of the calcium content of the standard Krebs-Henseleit solution (2.5 mM). The effects of various sensitizing treatments on the response to calcium restoration were determined by comparing the concentrations of calcium required to cause a response equal to 50 per cent of the maximum.

H. EXPRESSION OF RESULTS AND STATISTICAL COMPARISONS

The apparatus we used permitted the recording of responses from four strips in each experiment. When the experimental design did not require four different treatments, duplicate determinations of response were often made on two strips from the same spleen.

The values obtained were averaged before comparisons were made with other treatments.

Where comparisons were made between spleen strips from the same cat, or within the same spleen strip, the results were often treated as paired data. Statistical significance of the difference was determined by the t-test for paired observations (Goldstein, 1964), in which the mean difference is divided by its standard error. Where observations from different spleens were compared, statistical significance was calculated by Student's t-test, in which the difference between the mean value of each group is divided by the standard error of the pooled data. Mean values are reported with their standard errors.

RESULTS

SECTION ONE

RESULTS BEARING ON PRE-RECEPTOR EVENTS

A. PROTECTION AGAINST COCAINE-INDUCED SUPERSENSITIVITY

If cocaine causes supersensitivity by a competitive interaction with adrenergic receptors, with uptake sites for noradrenaline, or with some part of the adrenergic mechanism, it should be possible to prevent supersensitivity by saturation of the site of supersensitizing action with suitable substances which react with this site. Noradrenaline, tyramine, phentolamine, isoproterenol, pronethalol, acetylcholine and histamine were used as possible protecting agents against sensitization by cocaine. The experiments, with suitable controls, were done as illustrated in Table 1. The agonist used to test for sensitization was either adrenaline or noradrenaline in a dose selected to cause a response on the low portion of the dose-response curve. The responses after cocaine (10^{-5} g/ml) were calculated as a percentage of the control responses before cocaine. The results are shown in Figures 4-10, and are summarized in Table 2.

Noradrenaline

Noradrenaline was tested because it is the endogenous transmitter which combines with alpha receptors, and is markedly potentiated by cocaine. Figure 4 shows that time (column 1) and 5×10^{-5} g/ml of noradrenaline alone (column 4) had no effect on the test response. Exposure to 10 g/ml of cocaine for 5 minutes (column 2) clearly sensitized the test response, which was increased to 182.8 ± 21.6 per cent of control. When cocaine was added to the bath containing a large dose of noradrenaline which had been added 5 minutes before (column 3), the average increase in the

TABLE 2

THE EFFECT OF SOME SMOOTH MUSCLE STIMULANTS AND ANTAGONISTS ON SUPERSENSITIVITY INDUCED BY COCAINE IN ISOLATED SPLEEN STRIPS

Cocaine, 10^{-5} g/ml, was added to the bathing fluid for 5 minutes, alone, or in the presence of a protecting drug added 5 minutes earlier. The change in subsequent response to a low test dose of catecholamine was calculated as per cent of the control response in the same strip. These values, representing columns 2 and 3 in each of Figures 4-10, are shown in the table.

Test drug	Protecting drug g/ml	N*	Response after Cocaine (10^{-5} g/ml) per cent of control		P
			Unprotected	Protected	
Noradrenaline	Noradrenaline 1×10^{-5}	5	182.6 ± 21.6	158.6 ± 9.8	< 0.05
Noradrenaline	Tyramine 5×10^{-5}	4	318.8 ± 21.3	344.4 ± 44.8	NS
Adrenaline	Phentolamine 3×10^{-6}	4	376.5 ± 78.3	102.0 ± 21.6	< 0.005
Adrenaline	Isoproterenol 5×10^{-5}	4	290.8 ± 30.4	369.7 ± 80.5	NS
Adrenaline	Pronethalol 5×10^{-5}	4	263.5 ± 39.6	253.0 ± 33.7	NS
Adrenaline	Acetylcholine 5×10^{-5}	4	204.3 ± 25.7	237.5 ± 33.6	NS
Adrenaline	Histamine 5×10^{-5}	4	222.0 ± 13.7	220.8 ± 27.2	NS

* Number of experiments

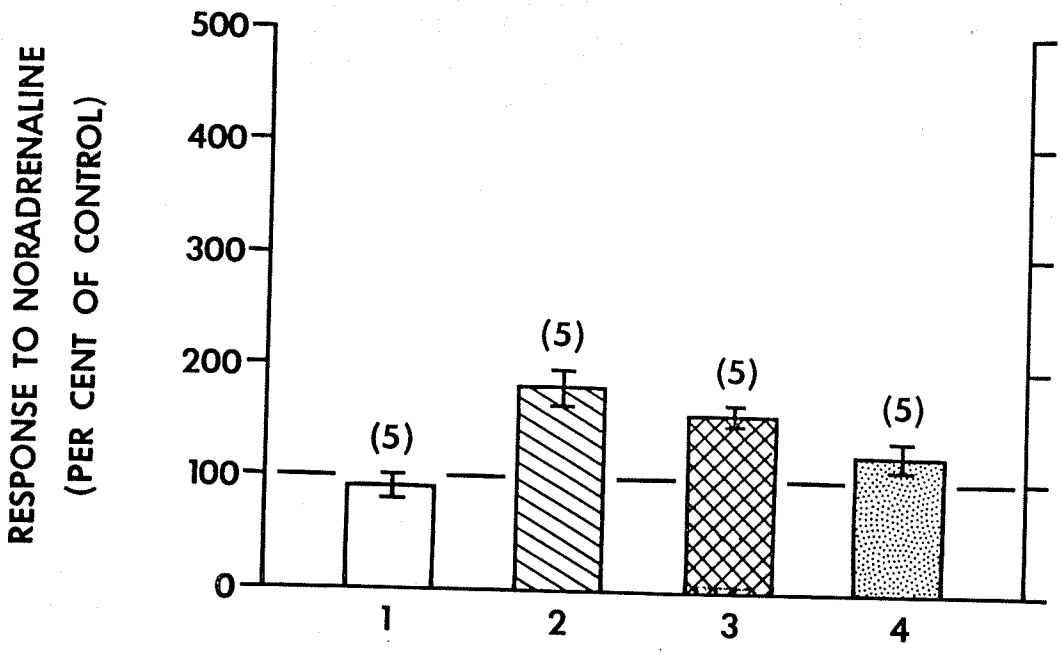

Fig. 4 THE EFFECT OF NORADRENALINE ON COCAINE-INDUCED SUPER-SENSITIVITY IN ISOLATED SPLEEN STRIPS.

Procedure is described in Table 1. Ordinate: average per cent change in noradrenaline contraction after treatment (no change = 100%). Columns: 1 = Time control, 2 = Cocaine (10^{-5} g/ml), 3 = Noradrenaline (10^{-5} g/ml) + cocaine (10^{-5} g/ml), 4 = Noradrenaline control (10^{-5} g/ml). Vertical lines show standard errors. Number of experiments in parentheses above columns.

Statistical comparisons 1 v 2: $P < .05$

2 v 3: $P > .10$

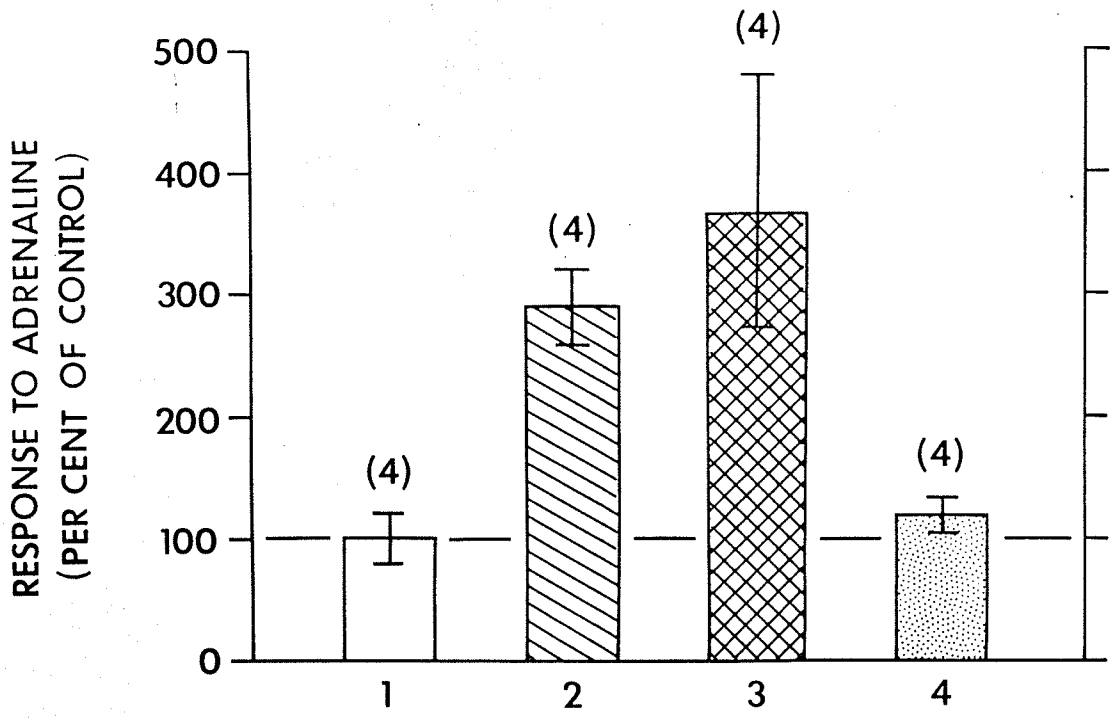

Fig. 5 THE EFFECT OF TYRAMINE ON COCAINE-INDUCED SUPERSENSITIVITY IN ISOLATED SPLEEN STRIPS.

Procedure is described in Table 1. Ordinate: average per cent change in adrenaline contraction after treatment (no change = 100%). Columns: 1 = Time control, 2 = Cocaine (10^{-5} g/ml), 3 = Tyramine (5 x 10^{-5} g/ml) + cocaine (10^{-5} g/ml), 4 = Tyramine control (5 x 10^{-5} g/ml). Vertical lines show standard errors. Number of experiments in parentheses above columns.

Statistical comparisons 1 v 2: P > .005

2 v 3: P > 0.5

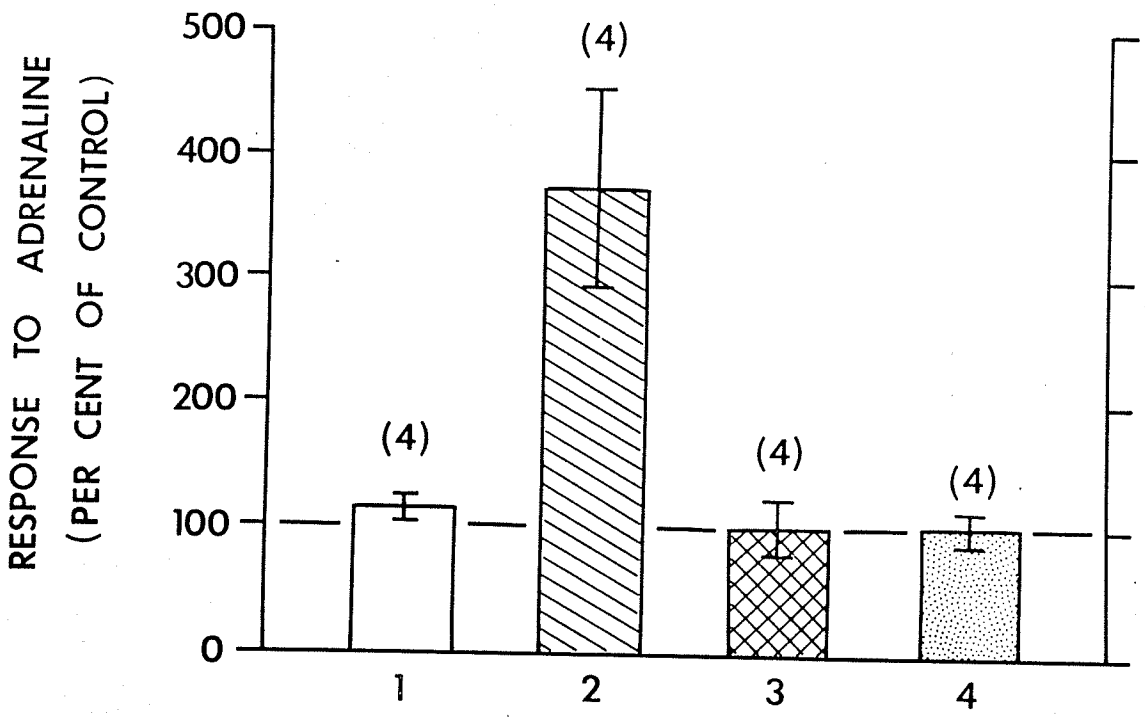

Fig. 6 THE EFFECT OF PHENTOLAMINE ON COCAINE-INDUCED SUPERSENSITIVITY IN ISOLATED SPLEEN STRIPS.

Procedure is described in Table 1. Ordinate: average per cent change in adrenaline contraction after treatment (no change = 100%). Columns: 1 = Time control, 2 = Cocaine (10^{-5} g/ml), 3 = Phentolamine (3×10^{-6} g/ml) + cocaine (10^{-5} g/ml), 4 = Phentolamine control (3×10^{-6} g/ml). Vertical lines show standard errors. Number of experiments in parentheses above columns.

Statistical comparisons 1 v 2: $P < .01$

1 v 3: $P > .9$

2 v 3: $P < .005$

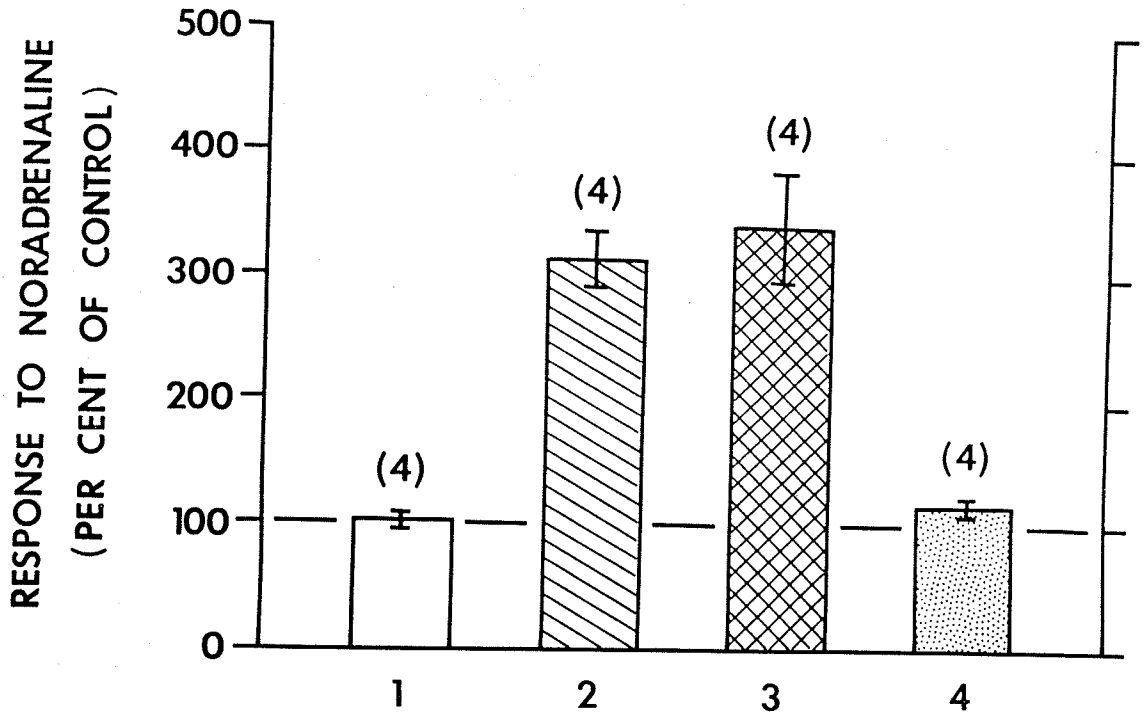

Fig. 7 THE EFFECT OF ISOPROTERENOL ON COCAINE-INDUCED SUPERSENSITIVITY IN ISOLATED SPLEEN STRIPS.

Procedure is described in Table 1. Ordinate: average per cent change in noradrenaline contraction after treatment (no change = 100%). Columns: 1 = Time control, 2 = Cocaine (10^{-5} g/ml), 3 = Isoproterenol (5×10^{-5} g/ml) + cocaine (10^{-5} g/ml), 4 = Isoproterenol control (5×10^{-5} g/ml). Vertical lines show standard errors. Number of experiments in parentheses above columns.

Statistical comparisons 1 v 2: $P < .001$

2 v 3: $P > 0.5$

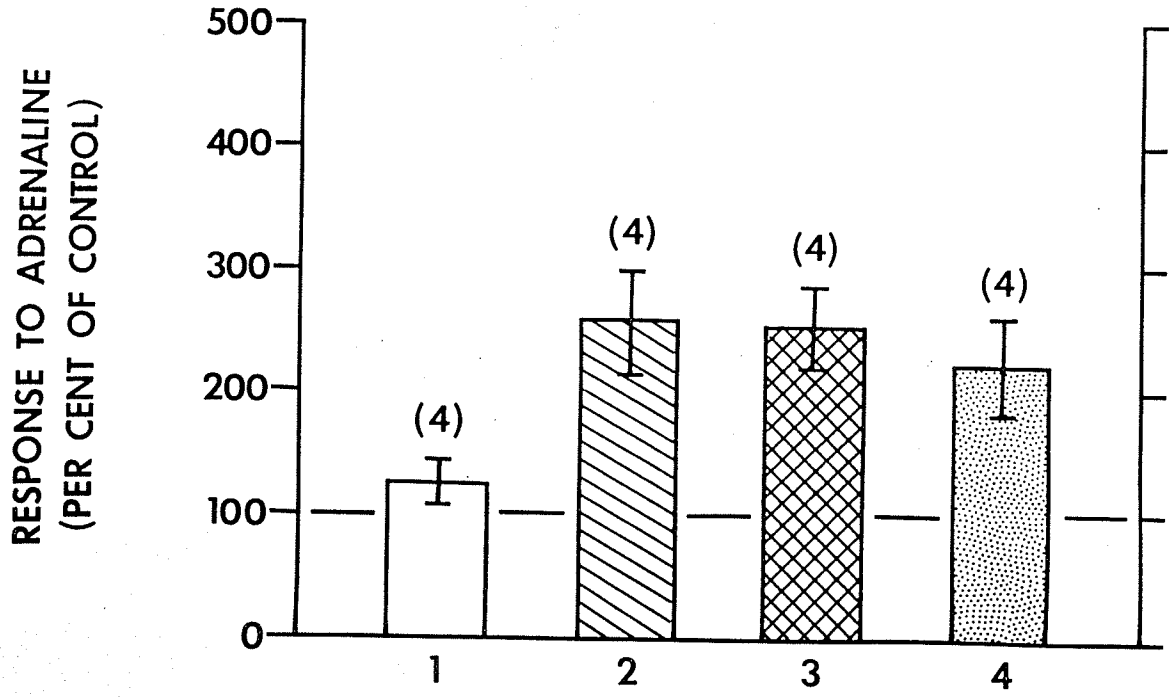

Fig. 8 THE EFFECT OF PRONETHALOL ON COCAINE-INDUCED SUPERSENSITIVITY IN ISOLATED SPLEEN STRIPS.

Procedure is described in Table 1. Ordinate: average per cent change in adrenaline contraction after treatment (no change = 100%). Columns: 1 = Time control, 2 = Cocaine (10^{-5} g/ml, 3 = Pronethalol (5×10^{-5} g/ml) + cocaine (10^{-5} g/ml, 4 = Pronethalol control (5×10^{-5} g/ml). Vertical lines show standard errors. Number of experiments in parentheses above columns.

Statistical comparisons 1 v 2: $P < .01$

1 v 3: $P < .01$

1 v 4: $P < .01$

2 v 3: $P > .8$

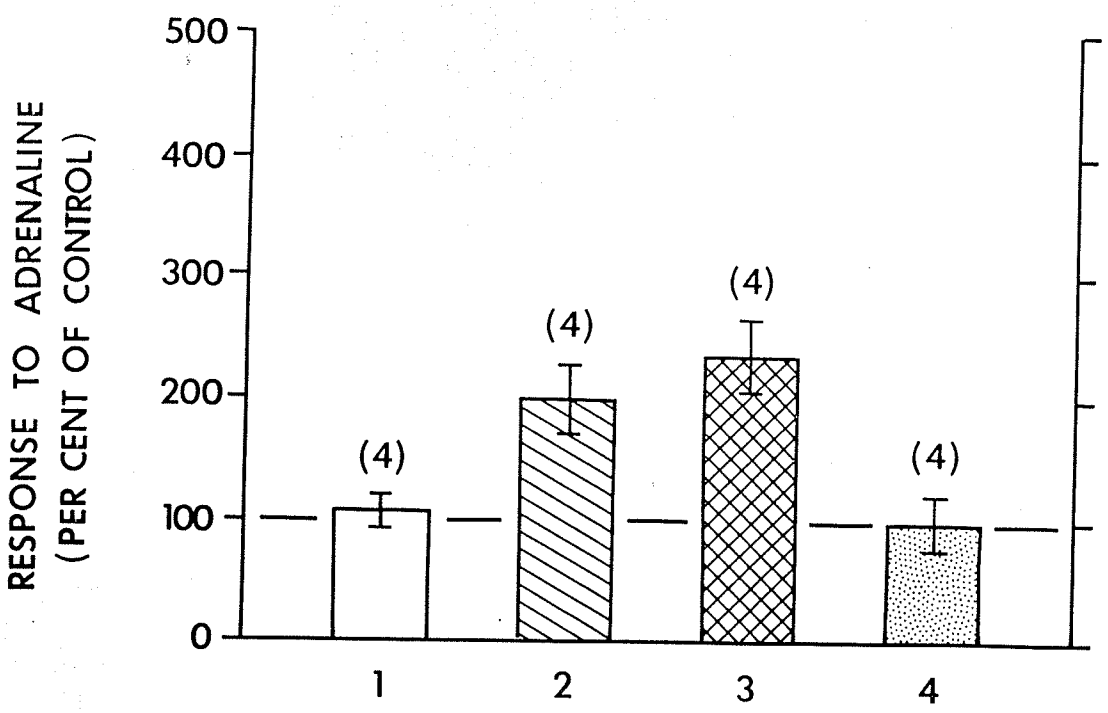

Fig. 9 THE EFFECT OF ACETYLCHOLINE ON COCAINE-INDUCED SUPERSENSITIVITY IN ISOLATED SPLEEN STRIPS.

Procedure is described in Table 1. Ordinate: average per cent change in adrenaline contraction after treatment (no change = 100%). Columns: 1 = Time control, 2 = Cocaine (10^{-5} g/ml), 3 = Acetylcholine (5 x 10^{-5} g/ml) + cocaine (10^{-5} g/ml), 4 = Acetylcholine control (5 x 10^{-5} g/ml). Vertical lines show standard errors. Number of experiments in parentheses above columns.

Statistical comparisons 1 v 2: $P < .01$

2 v 3: $P > .9$

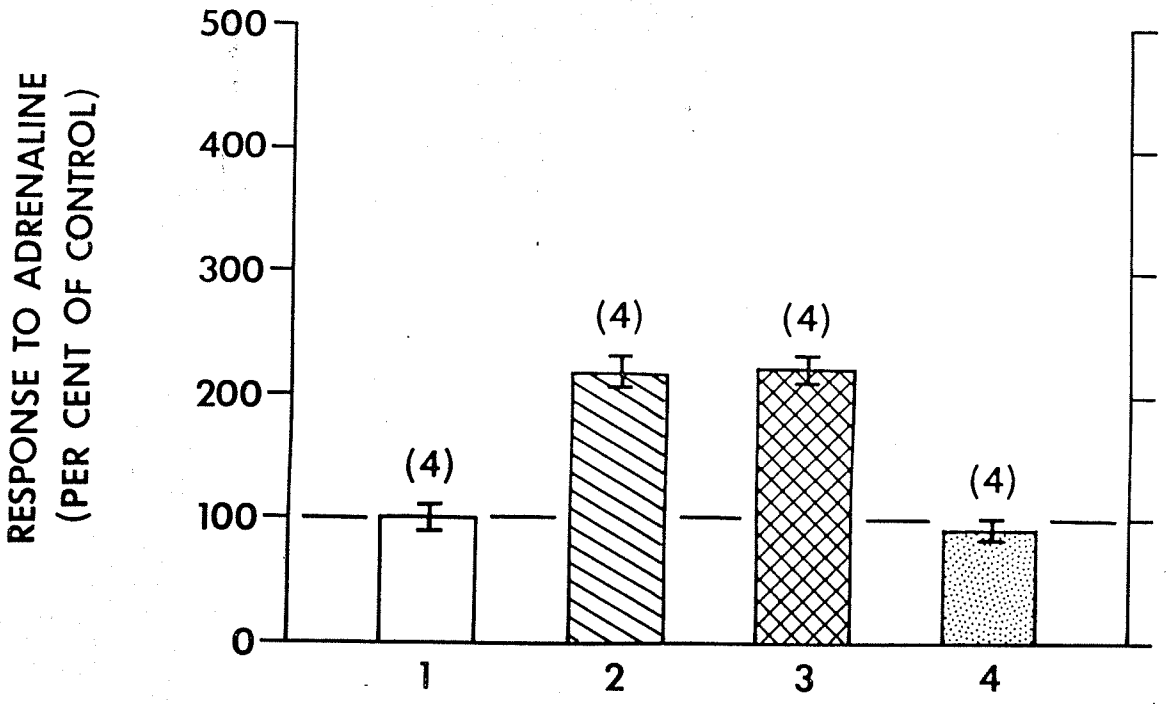

Fig. 10 THE EFFECT OF HISTAMINE ON COCAINE-INDUCED SUPERSENSITIVITY IN ISOLATED SPLEEN STRIPS.

Procedure is described in Table 1. Ordinate: average per cent change in adrenaline contraction after treatment (no change = 100%). Columns: 1 = Time control, 2 = Cocaine (10^{-5} g/ml), 3 = Histamine (5×10^{-5} g/ml) + cocaine (10^{-5} g/ml), 4 = Histamine control (5×10^{-5} g/ml). Vertical lines show standard errors. Number of experiments in parentheses above columns.

Statistical comparisons 1 v 2: $P < .001$

2 v 3: $P > .8$

test response was slightly reduced to 158.6 ± 9.8 per cent of control. This difference was not statistically significant ($0.2 > P > 0.1$, Table 2).

Tyramine

Tyramine is a sympathomimetic amine with little direct action on alpha receptors (Trendelenburg, 1961). Responses to tyramine are normally mediated by the release of noradrenaline from its binding sites (Burn & Rand, 1960; Stjärne, 1961), and this effect is blocked by cocaine (Tainter & Chang, 1927; Iversen & Whitby, 1962; Farrant, 1963). The nature of this block is competititve, since it is overcome by increasing the dose of tyramine (Hertting, Axelrod & Patrick, 1961). Tyramine was therefore tested for its ability to prevent cocaine supersensitivity (Fig. 5). Neither time nor the large dose of tyramine (5×10^{-5} g/ml) altered the test response (colmuns 1 & 4). Cocaine alone increased the test response to 318.8 ± 21.3 per cent (column 2) and treatment with tyramine did not reduce this increase (column 3). The apparent increase shown in column 3 is not statistically significant. Thus tyramine did not protect against cocaine supersensitivity in our experiments.

Phentolamine

This drug is a competitive adrenergic blocking agent with high affinity for alpha receptors (Nickerson, 1949). In the concentrations we used (3×10^{-6} g/ml) it caused no contraction of the isolated spleen strip. Its effect on cocaine supersensitivity is shown in Figure 6. The test response did not change with time or

after phentolamine alone (columns 1 & 4). Cocaine alone increased the test response to 376.5 ± 78.3 per cent of control (column 2). In the presence of phentolamine, however, the test response after cocaine was 102.0 ± 21.6 per cent of control (column 3). This is not significantly different from the time control value, and is significantly less than the change due to cocaine alone ($P < .005$, Table 2). Thus phentolamine afforded complete protection against the sensitizing effect of cocaine in the spleen strip.

Isoproterenol

Isoproterenol has no appreciable affinity for the catecholamine uptake process (Burgen & Iversen, 1965). In most tissues which respond to adrenergic drugs, it is most potent on beta receptors (Ahlquist, 1948). Bickerton (1963) showed that in the cat spleen low doses of isoproterenol have little beta receptor activity, and that higher doses stimulate alpha receptors. This suggests that the spleen has a limited number of beta receptors. However, this does not rule out the possibility that cocaine interacts with a site resembling the beta recepter. We therefore tested the ability of isoproterenol to protect against cocaine supersensitivity (Fig. 7). Test responses after time or isoproterenol (5×10^{-5} g/ml) were unchanged (columns 1 & 4). The test response was increased to 290.8 ± 30.4 per cent by cocaine alone (column 2) and isoproterenol did not alter this effect significantly (column 3).

Pronethalol

Pronethalol is a potent beta adrenergic blocking agent with much less intrinsic activity than its prototype, dichloroisoproterenol, and even in high concentrations, without blocking

action on alpha receptors in the uterus and ear vessels of the rabbit (Black & Stephenson, 1962).

Figure 8 shows the results of protection experiments with pronethalol (5×10^{-5} g/ml). Noradrenaline responses were increased after cocaine (column 2) and after pronethalol (column 4). In both cases the increases compared to time control (column 1) were significant ($P < .01$). The duration of the potentiation after pronethalol was as great as that after cocaine, making it impossible to separate the direct action of pronethalol from any effect it may have had to alter cocaine potentiation. It is therefore impossible to place any interpretation on these results.

Acetylcholine and Histamine

Both acetylcholine and histamine stimulate the smooth muscle of the spleen through receptors which are pharmacologically distinct from adrenergic alpha and beta receptors (Innes, 1961). They were tested as protecting drugs in an attempt to reveal possible unspecific protection independent of the adrenergic mechanism. The results with acetylcholine and histamine were similar (Figs. 9 & 10). The test responses did not change due to time or protecting drug along (columns 1 & 4). Cocaine equally increased the test response over control whether acetylcholine or histamine was present or not (columns 2 & 3).

B. THE EFFECT OF COCAINE ON RESTORATION OF THE NORADRENALINE RESPONSE AFTER PHENTOLAMINE

In the protection experiments with phentolamine, it was assumed that cocaine did not alter the duration of the phentolamine block. However, if cocaine decreased the rate of dissociation of

phentolamine from the alpha receptors this would prolong alpha receptor blockade. Test responses to adrenaline would return to normal sooner in the phentolamine control strips, and the persisting antagonism in the experimental strips would create the illusion of protecting against potentiation by cocaine.

To test this possibility we studied the effect of cocaine on the duration of the phentolamine block. First a maximal steady-state contraction was obtained with noradrenaline. Phentolamine was then added to cause a substantial decrease in the steady-state response. When the drugs were removed and the maximal contracting dose of noradrenaline was restored to the tissue, the rate of recovery from the antagonism could then be determined. The rate at which the tissue recovered from the block was regarded as a measure of the dissociation of phentolamine from the alpha receptors. The effect of cocaine was determined by adding it to the bath 5 minutes before washing out the phentolamine, similar to the procedure followed in the protection experiments. Figure 11 shows representative tracings from one of two such experiments. Four strips from the same spleen were used in each experiment. All were first exposed to noradrenaline (1.1×10^{-4} g/ml) to produce steady-state contractions. One strip was given no further treatment and established the persistence of the steady-state contraction (not shown in Fig.). Phentolamine to a total of 1.1×10^{-4} g/ml was added to the other 3 strips. One strip (Fig. 11a) served as a control for the persistence of the antagonism by phentolamine, and was therefore given no further treatment. In the two

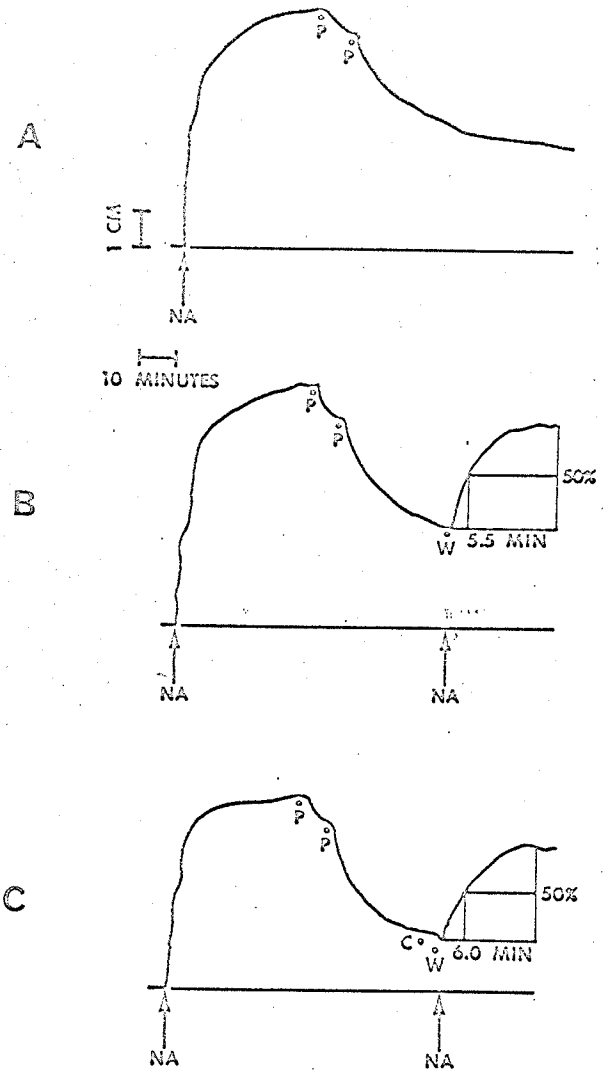

Fig. 11 THE EFFECT OF COCAINE ON THE DURATION OF PHENTOLAMINE BLOCK IN SPLEEN STRIPS.

Noradrenaline (NA), 1.55 x 10^{-4} g/ml, was first added to all strips. At P, phentolamine was added in all tissues (total 1.1 x 10^{-4} g/ml). In C, cocaine, 10^{-5} g/ml, was added at C. Five minutes later at W strips B and C were washed and noradrenaline (1.55 x 10^{-4} g/ml) was immediately added. The time to 50% of the maximum contraction was calculated as shown.

remaining strips the phentolamine was washed out after the maximum reduction of the contraction had been reached, but in one of the two strips cocaine (10^{-5} g/ml) was added to the bath 5 minutes before the removal of the phentolamine. Noradrenaline (1.1×10^{-4} g/ml) was added to both strips immediately after the wash. The time taken to reach 50 per cent of the subsequent contraction was taken as a measure of the recovery time (Fig. 11, b & c).

The 50 per cent rise times in the two cocaine treated tissues were 6.0 and 4.0 minutes, compared to 5.5 and 3.5 minutes for their respective controls. This might indicate a slight effect of cocaine to decrease phentolamine dissociation; however, we did not regard this as adequate to account for the effect of phentolamine in the protection experiments.

C. THE EFFECT OF COCAINE ON STEADY-STATE SUBMAXIMUM RESPONSES

In studies of potentiation of an agonist by cocaine, the agonist is usually allowed to act in the presence of a dose of cocaine added beforehand. This response is compared with a previous response without cocaine. Since cccaine is thought by many to cause supersensitivity by preventing uptake, we wanted to study the effect of cocaine after uptake from a dose of noradrenaline had already occurred. A single dose of noradrenaline which would fall in the lower region of the dose-response curve was added to each of 4 spleen strips from two cats. After a steady-state contraction was obtained 3×10^{-6} g/ml of cocaine was added. Cocaine markedly increased the contraction in all 4 strips (Fig. 12). Similar steady-state responses to isoproterenol were also clearly potentiated by cocaine in another 4 strips from the same two spleens (Fig. 13).

SECTION TWO

RESULTS BEARING ON RECEPTOR AFFINITY

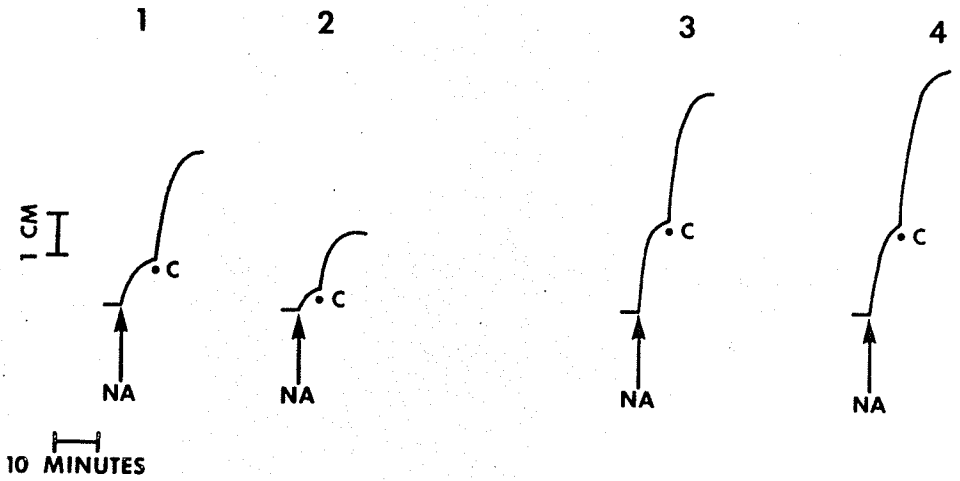

Fig. 12 POTENTIATION BY COCAINE OF STEADY-STATE CONTRACTIONS TO NORADRENALINE.

Tracings of contractions to small doses of noradrenaline (NA) (1 & 2 = 6 x 10^{-7} g/ml, 3 & 4 = 3 x 10^{-6} g/ml) in 4 strips from 2 cat spleens. Cocaine (3 x 10^{-6} g/ml) was added at C.

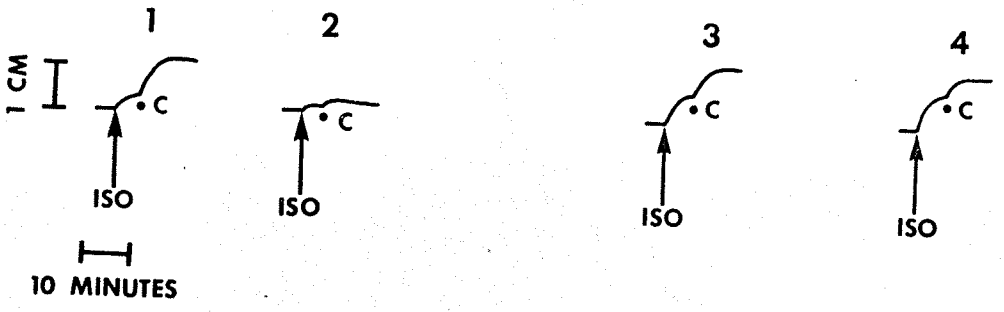

Fig. 13 POTENTIATION BY COCAINE OF STEADY-STATE CONTRACTIONS TO ISOPROTERENOL.

Tracings of contractions to small doses of isoproterenol (ISO) (1 & 2 = 3 x 10^{-5} g/ml, 3 & 4 = 6 x 10^{-5} g/ml) in 4 strips from 2 cat spleens. Cocaine (3 x 10^{-6} g/ml) was added at C.

The studies in cocaine-induced supersensitivity reported in SECTION ONE indicated that the sensitization may involve factors other than catecholamine uptake. In the Introduction (page 11) we raised the theoretical possibility that supersensitivity could be caused by changes in the properties of the active receptors. In this section, experiments are described in which receptor antagonists were used to evaluate changes in affinity between normal spleen strips and strips which have been made supersensitive by chronic denervation, by treatment with reserpine, or by cocaine. Reasons for using antagonists in these experiments are discussed on page 32.

A. SENSITIVITY OF THE DENERVATED SPLEEN

In four experiments strips from half spleens which had been denervated were compared with control strips, both from the innervated half of the same spleen(operated controls) and from fully innervated spleens from other cats (normal controls). Figure 14 shows dose-response curves to noradrenaline from a typical experiment. In all 4 experiments the curves for denervated strips were to the left of those for both operated and normal control strips. The dose-response curves from the operated control strips were to the left of those from the normal controls in 3 of the 4 experiments. However, this difference was always considerably less than the shift due to denervation. Tyramine, 10^{-5} g/ml, was always tested at the beginning of an experiment with denervated spleen, and in all experiments failed to cause a response in the denervated strips. Responses were always seen in the control strips,

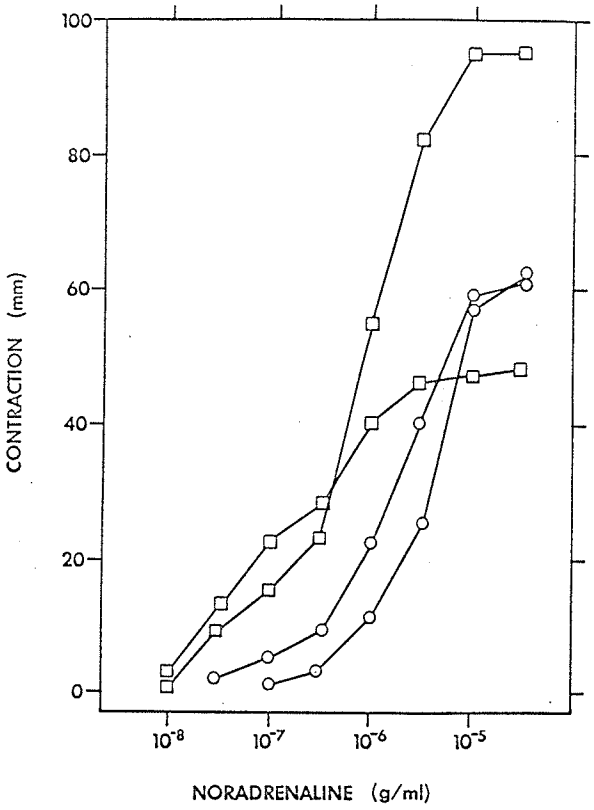

Fig. 14 THE EFFECT OF DENERVATION OF CAT SPLEEN ON THE DOSE-RESPONSE CURVES TO NORADRENALINE.

Two strips from a normal control spleen, O—O are compared with 2 strips from a denervated spleen, □—□ .

but the contraction was always smaller in the operated control than in the normal one. These observations suggested that the "innervated" half of an operated spleen was in fact partially denervated. Therefore, only normal controls were used in subsequent experiments with denervated spleens.

B. SENSITIVITY OF SPLEEN FROM RESERPINE-TREATED CATS

In 8 experiments dose-response curves were obtained for strips of normal spleen and for strips from cats given 1 mg/kg of reserpine 16-24 hours earlier. Figure 15 shows dose-response curves from a typical experiment in which a normal strip is compared with one from a reserpine treated cat. In this and all other experiments, the threshold dose of noradrenaline was less in strips from the reserpine treated animal, and the curve was shifted to the left throughout the entire dose range tested, with the exception, in a few strips, of the very highest doses tested. Tyramine, (10^{-5} g/ml), tested at the beginning of each experiment, caused a 10-20 mm contraction in the normal strips, but failed to contract the reserpine treated ones.

C. PHENTOLAMINE ANTAGONISM

pA_{10} values for phentolamine antagonism of noradrenaline were determined in 9 spleen strips from 4 cats given reserpine (1 mg/kg) 16-24 hours earlier, in 7 strips from 4 spleens denervated 14-17 days earlier, and in 11 control strips from 6 normal spleens (Table 3). The unit dose of noradrenaline used in each strip was from 5×10^{-8} to 10^{-6} g/ml, and was selected to cause a response on the lower half of the dose-response curve. For each strip the

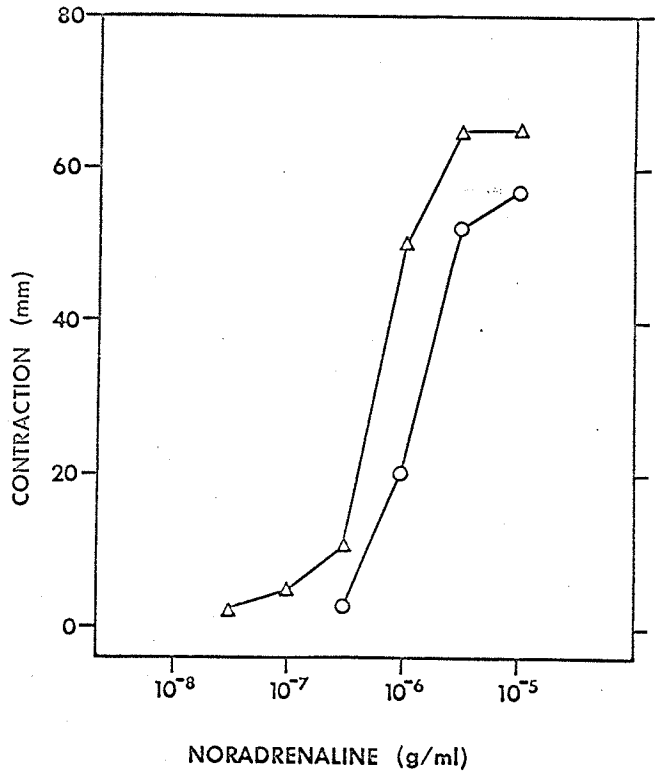

Fig. 15 THE EFFECT OF TREATMENT WITH RESERPINE ON THE DOSE-RESPONSE CURVE FOR NORADRENALINE IN CAT SPLEEN.

Normal control spleen strip, O—O ; strip from a reserpine-treated cat, Δ——Δ. Reserpine (1 mg/kg) was given 16-24 hours before the experiment.

TABLE 3

THE EFFECT OF DENERVATION AND TREATMENT WITH RESERPINE ON THE pA_{10} FOR PHENTOLAMINE ANTAGONISM OF NORADRENALINE (5×10^{-8} to 10^{-6} g/ml)

For reserpine treatment 1 mg/kg was given 16 - 24 hours before the experiment. When more than one strip from a spleen was tested, the average value for each spleen is given.

	pA_{10}		
	Control	Denervation	Reserpine
	5.85	5.70	5.56
	4.81	5.24	5.47
	5.46	5.81	5.55
	5.21	5.30	5.46
	6.74		6.92
	4.78		
Mean $\pm$ S.E.	5.47 $\pm$ 0.30	5.51 $\pm$ 0.11	5.79 $\pm$ 0.28

dose of phentolamine which would make the response to a tenfold increase over the unit dose of adrenaline equal the response to the unit dose was calculated and is tabulated as the pA_{10}. (For details of the calculation, see page 41). The mean pA_{10} value for strips from reserpine treated cats (5.79 ± 0.28) appeared slightly greater than the control mean (5.47 ± 0.30). However, the differences were not statistically significant ($P > .60$). The pA_{10} values for phentolamine in denervated spleens similarly did not differ from the values in normal controls. Thus, neither denervation nor treatment with reserpine caused any apparent change in the ability of phentolamine to antagonize noradrenaline in spleen strips.

D. PHENOXYBENZAMINE ANTAGONISM

1. Dose Ratios

Dose-ratios for phenoxybenzamine antagonism of noradrenaline were determined in 12 normal control strips, in 8 chronically denervated strips, and in 4 strips from reserpine treated cats.

Denervated spleens

In preliminary experiments, a dose of phenoxybenzamine of 5×10^{-8} g/ml with an exposure time of 5 minutes was established as adequate to cause a definite shift of the dose-response curve to the right. This was usually associated with a reduction of the maximum response, and deviation from parallel of the upper region of the curve. The dose-ratio at 50 per cent of the reduced maximum was therefore determined as described on page 39 . Figure 16 shows the typical effect of phenoxybenzamine on control and denervated strips; the shift of the dose-response curve to the right due to phenoxybenzamine is greater in the denervated spleen. Table 4 shows the

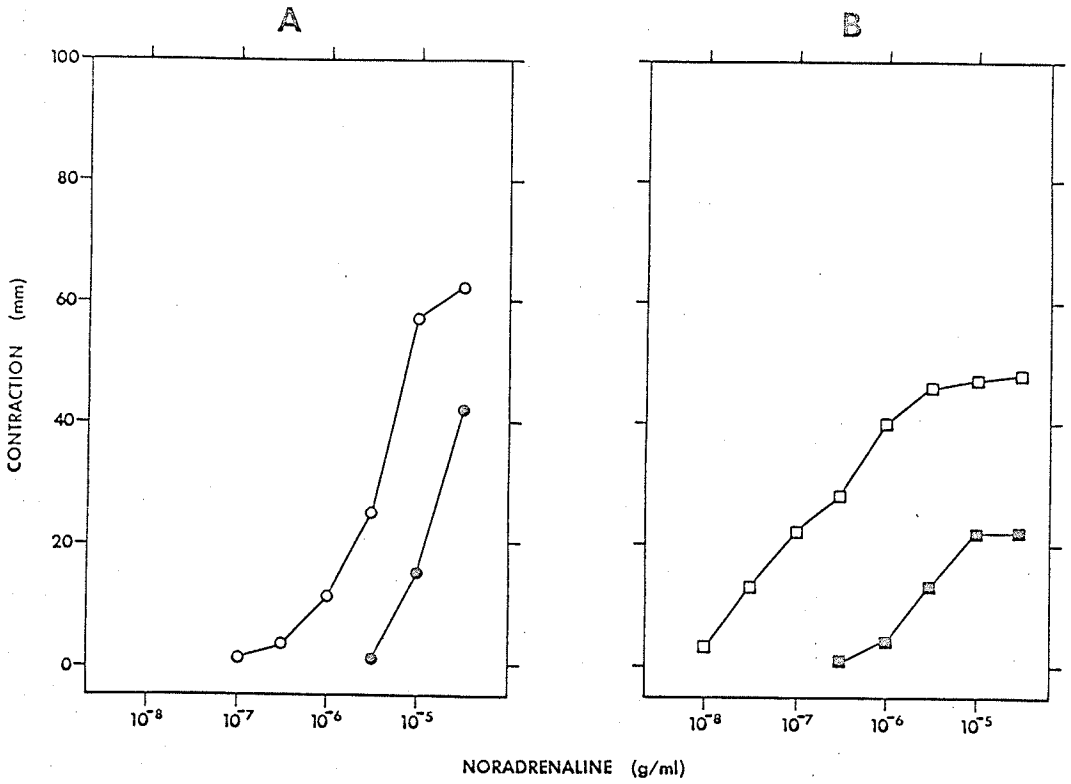

Fig. 16 THE EFFECT OF PHENOXYBENZAMINE ON RESPONSES OF NORMAL AND DENERVATED SPLEEN TO NORADRENALINE.

A- normal control spleen: before phenoxybenzamine ○—○; after phenoxybenzamine ◎—◎. B- denervated spleen: before phenoxybenzamine □—□; after phenoxybenzamine ▣—▣. The dose of phenoxybenzamine was 5 x 10-8 g/ml.

TABLE 4

THE EFFECT OF DENERVATION ON DOSE-RATIOS FOR ANTAGONISM OF NORADRENALINE BY PHENOXYBENZAMINE IN THE ISOLATED SPLEEN STRIP

Dose-response curves to noradrenaline were obtained before and after 5 minute exposure to phenoxybenzamine (5×10^{-8} g/ml). Dose-ratios calculated at 50% of the maximum response after phenoxybenzamine are shown in the table. Two strips of each spleen were tested and the values averaged to obtain the figures in the table.

	Control	Denervated
	4.74	33.55
	4.74	16.27
	5.32	10.61
	3.91	5.05
Mean ±S.E.	4.68 ± 0.29	16.38 ± 6.16

dose-ratios from all experiments. The mean dose-ratio of 16.38 ± 6.16 for the denervated strips represents slightly more than a 3 fold greater antagonism than in the control strips.

Spleens from reserpine treated cats

For these experiments a dose of phenoxybenzamine of 2.5 x 10^{-8} g/ml with an exposure time of 5 minutes was used. Dose-response curves to noradrenaline were determined as in the experiments with denervated spleens. There was no apparent difference between reserpine and control strips in the shift caused by phenoxybenzamine (Fig. 17). Table 5 shows the dose-ratios from all 4 strips. The mean dose-ratios for strips from reserpine-treated cats and for normal controls did not differ significantly.

2. Decay of the Steady-State Response

When phenoxybenzamine, 10^{-7} to 10^{-6} ml, is added to a spleen strip which has been maximally contracted with noradrenaline, the contraction height decays exponentially and approaches a new level which is usually from 20 to 50 per cent of the original height. If it is assumed that the new level of response represents the removal of a portion of the alpha receptors, the rate of decay may be used as an index of the rate of combination of phenoxybenzamine with the resceptors. Six experiments were done to determine whether cocaine affected the rate of decay due to phenoxybenzamine. In each experiment, two pairs of strips from the same spleen were maximally contracted with noradrenaline (10^{-4} g/ml). After a steady-state was reached, usually about 30 min, cocaine (10^{-6} or 10^{-5} g/ml) was added to one strip of each pair; five minutes later phenoxybenzamine (10^{-7}

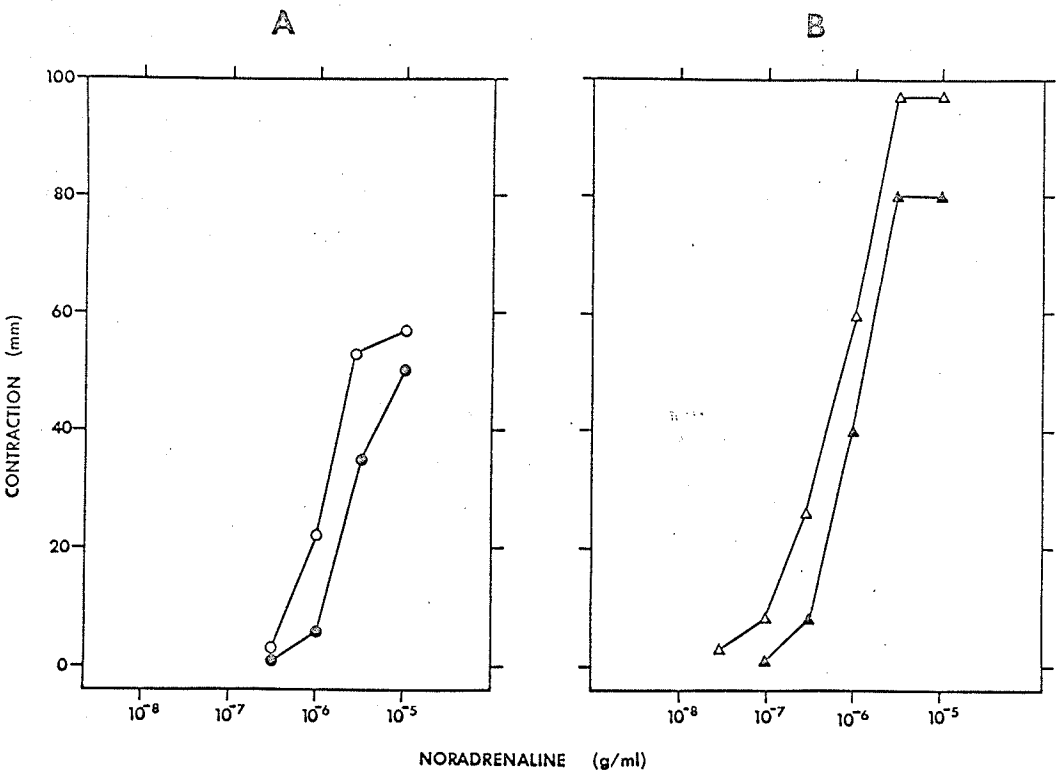

Fig. 17 THE EFFECT OF PHENOXYBENZAMINE TREATMENT ON RESPONSES OF NORMAL AND RESERPINE-TREATED SPLEEN TO NORADRENALINE.

A- normal spleen: before phenoxybenzamine O—O ; after phenoxybenzamine ◎—◎. B- spleen of reserpine treated cat : before phenoxybenzamine Δ—Δ ; after phenoxybenzamine ▲—▲. The dose of phenoxybenzamine was 2.5×10^{-6} g/ml. Reserpine (1 mg/kg) was given 16-24 hours before the experiment.

TABLE 5

THE EFFECT OF RESERPINE TREATMENT ON DOSE-RATIOS FOR ANTAGONISM OF NORADRENALINE BY PHENOXYBENZAMINE IN THE ISOLATED SPLEEN STRIP

Dose-response curves to noradrenaline were obtained before and after 5 minute exposure to phenoxybenzamine (2.5×10^{-8} g/ml). Dose-ratios calculated at 50% of the maximum response after phenoxybenzamine are shown in the table. For reserpine treatment 1 mg/kg was given 16 - 24 hours before the experiment.

	Control	Reserpine
	1.78	1.78
	1.20	1.20
	1.59	1.59
	1.59	1.78
Mean $\pm$ S.E.	1.54 ± 0.12	1.59 ± 0.14

or 10^{-6} g/ml) was added to both. The times for reduction of the response to 25, 50 and 75 per cent of the total decay to the final steady-state, measured as shown in Figure 3 page 46 , are given in Table 6. In all cases, cocaine decreased the time required for 25, 50, and 75 per cent decay, thus indicating an increase in the decay rate. With paired data analysis based on the average of the values within each spleen, the difference was significant ($p < .05$) at each level of decay. Figure 18 confirms the linear logarithmic relation between time and decay which is expected of an exponential function. The parallelism between the two curves indicates that the effect of cocaine bears a direct relation to the rate of decay.

TABLE 6

THE EFFECT OF COCAINE (10^{-6} or 10^{-5} g/ml) ON THE RATE OF REDUCTION BY PHENOXYBENZAMINE (10^{-7} - 10^{-6} g/ml) OF THE STEADY-STATE MAXIMUM RESPONSE TO NORADRENALINE (10^{-4} g/ml)

In each of 6 experiments phenoxybenzamine was added to 4 maximally contracted strips from the same spleen. Two of these were given cocaine 5 minutes earlier, and two others were controls. The time required for the contraction to fall to 25, 50 and 75 per cent of the new level was recorded. The values obtained for each pair of strips were averaged to obtain the figures in the table.

	25% time (min.)			50% time (min.)			75% time (min.)		
	Control	Cocaine	Difference	Control	Cocaine	Difference	Control	Cocaine	Difference
	3.50	1.50	2.00	5.00	4.25	1.25	8.25	8.00	0.25
	4.75	3.00	1.75	12.25	8.00	4.25	29.75	18.50	11.25
	5.00	3.00	2.00	22.25	8.25	14.00	52.75	24.50	28.50
	2.00	2.00	0	3.50	3.50	0	7.75	6.65	1.00
	4.50	3.75	0.75	13.50	12.00	1.50	38.50	33.50	5.00
	3.75	2.25	1.50	7.75	5.50	2.25	18.75	12.75	6.00
Mean	3.92	2.58	1.33	10.71	6.92	3.88	25.96	17.33	8.67
±S.E.	0.45	0.33	0.33	2.81	1.29	2.10	7.27	4.22	4.28

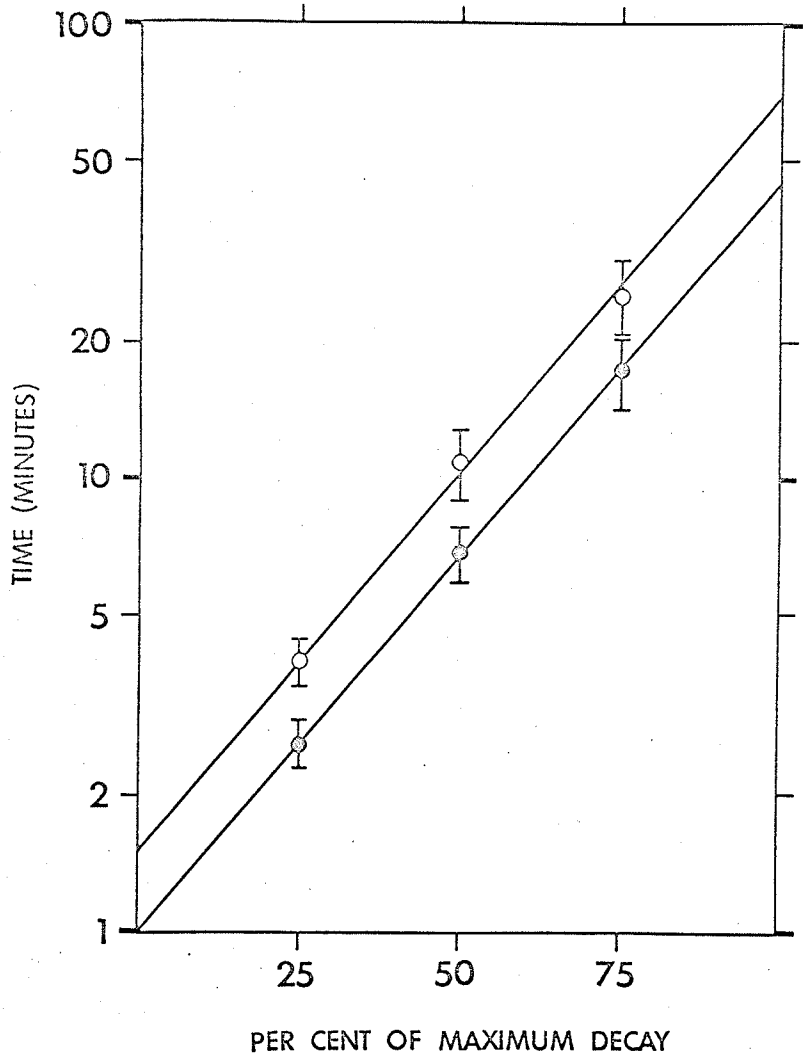

Fig. 18 EFFECT OF COCAINE ON THE RATE OF PHENOXYBENZAMINE-INDUCED DECAY OF MAXIMUM CONTRACTIONS TO NORADRENALINE IN SPLEEN STRIPS.

Procedure is described in Table 6. Ordinate: time in minutes (log scale). Abcissa: per cent decay of contraction. Control strips (no cocaine) O—O ; cocaine (10^{-6} or 10^{-5} g/ml) added before phenoxybenzamine (10^{-7} to 10^{-6} g/ml) ◉—◉ . Vertical lines show standard errors.

Statistical analysis: 25%, $\underline{P} < .01$

50%, $\underline{P} = .05$

75%, $\underline{P} < .05$

SECTION THREE

RESULTS BEARING ON EFFICACY

A. MAXIMUM RESPONSES

In 1949 Cannon & Rosenblueth discussed supersensitivity in relation to tissue "reactivity" and concluded that chronic denervation did not change the maximum possible response of effectors. As discussed in the Introduction (page 6), procedures causing supersensitivity resembling that of chronic denervation have been assumed not to change maximum responses. The experiments reported in this section were designed to examine this assumption and its implications more closely.

1. Maximum Response with Supersensitivity Induced before Exposure to Agonist

a. Noradrenaline

i. Chronic denervation

The effects of chronic denervation and reserpine treatment on responsiveness of the strips could not be determined within a single strip; and responses of treated strips were therefore compared with responses of strips from untreated cats. However, the experiments were generally planned so that maximum responses to noradrenaline for supersensitivite and normal tissues were determined simultaneously in an attempt to minimize the factors causing variation between strips. As stated on page 35 , all spleen strips were carefully cut to the same initial length (20 mm). The change in length after the strip stretched in the bath (10-15 mm) was not always the same for strips from different spleens, but there was no correlation between treatment and change in length. Since it seemed to be a random effect unrelated to treatment, we did not regard this as a complication in the study of maximum responses. Table 7 shows

the results of 5 experiments in which maximum responses of 10 strips from 5 chronically denervated spleens were compared with the maximum responses of a similar number of normal spleen strips. The average values for each group, shown in the table, are not significantly different, and indicate that chronic denervation causes no change in maximum response.

ii. Reserpine

Maximum responses to noradrenaline were obtained in spleen strips from 7 reserpine treated cats and from 17 normal animals. The average value for each spleen is shown in Table 8. The average maximum response in the reserpine group (86.1 ± 8.2 mm) is 14.5 mm greater than the average for the normal control group (71.6 ± 1.7 mm). This difference is statistically significant ($P < .05$).

iii. Cocaine

Full Maximum

Cumulative dose-response curves for noradrenaline were obtained before and after cocaine 10^{-5} g/ml in 4 spleen strips from 2 cats. In all of the 4 strips cocaine produced the characteristic potentiation of lower doses of noradrenaline. However, none of the strips showed an increase in the maximum response to noradrenaline. Figure 19 shows the effect of cocaine in 2 such experiments.

Reduced Maximum

Nickerson (1956) and Stephenson (1956) have shown that many agonists do not need to occupy all available receptors

TABLE 7

THE EFFECT OF DENERVATION ON THE MAXIMUM RESPONSE TO NORADRENALINE IN ISOLATED SPLEEN STRIPS

Maximum responses to noradrenaline (10^{-4} g/ml) were determined as part of full dose-response curves. The figures in the table are average values for 2 strips from each spleen.

	Control	Denervated
	(mm)	(mm)
	34.0	38.5
	42.5	41.5
	43.0	41.5
	52.5	41.5
	61.5	71.5
Mean ± S.E.	46.7 ± 4.71	46.9 ± 14.66

TABLE 8

THE EFFECT OF TREATMENT WITH RESERPINE ON THE MAXIMUM RESPONSE TO NORADRENALINE IN ISOLATED SPLEEN STRIPS

Maximum responses to noradrenaline (10^{-4} g/ml) were determined as part of full dose-response curves. Each figure in the table is the average value for 2 or more strips from one spleen.

Control		Reserpine
(mm)		(mm)
39.5		59.0
83.5		81.0
77.5		98.5
68.0		80.0
80.0		117.5
75.5		104.0
110.0		62.5
68.5	Mean ± S.E.	86.1 ± 8.2
94.8		
75.5		
63.5		
82.5		
75.0		
69.0		
34.0		
54.8		
66.3		
Mean ± S.E. 71.6 ± 1.7		

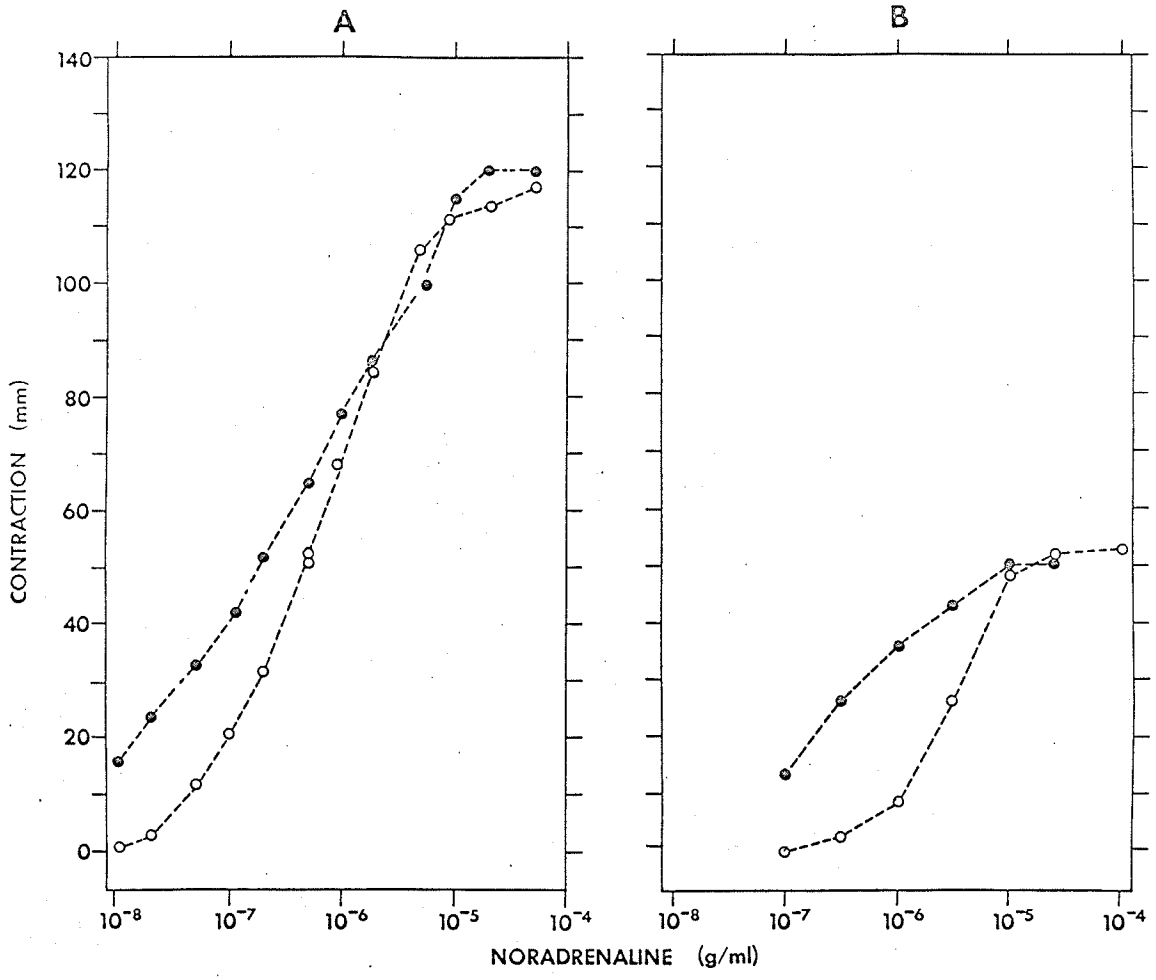

Fig. 19 EFFECT OF COCAINE ON DOSE-RESPONSE CURVES FOR NORADRENALINE.

Each panel represents a separate experiment. Before cocaine, O—O ; after cocaine, (10^{-5} g/ml), ●——● .

to cause a maximum tissue response. Furchgott (1955) provided evidence that this is the case for the alpha receptor response to adrenaline in rabbit aortic strips. When such "spare receptors" (Stephenson, 1956) exist, the maximum response is limited by factors other than the potency of the activated receptors. Thus an increase in the quantum of the response provided by each noradrenaline-activated receptor would not be seen as an increase in the maximum response. Experiments were designed to test the effect of cocaine on maximum responses to noradrenaline when all the spare receptors have been removed by irreversible combination with phenoxybenzamine. In this situation, any change in the efficacy of the drug-receptor complex should result in an increased height of the maximum contraction. Dose-response curves were established in 12 spleen strips from 12 cats before and 30 minutes after 5 minute exposure to phenoxybenzamine, 5×10^{-8} g/ml. The maximum response after phenoxybenzamine was always 15 to 40 per cent of the response to the largest dose of noradrenaline tested before. Thirty minutes after removal of the phenoxybenzamine, cocaine, 3×10^{-6} g/ml, was added for 5 minutes, and the maximum response to noradrenaline again tested. Because of the length of the experiments full dose-response curves were determined in only 4 strips; Figure 20 illustrates one experiment. In the other 8 strips, only doses in the upper range were tested before phenoxybenzamine, and full dose-response curves were tested after phenoxybenzamine. After cocaine, a single large dose of noradrenaline (5×10^{-5} g/ml) was added to see if the maximum response had changed. In all 12 strips cocaine failed to increase the maximum response observed

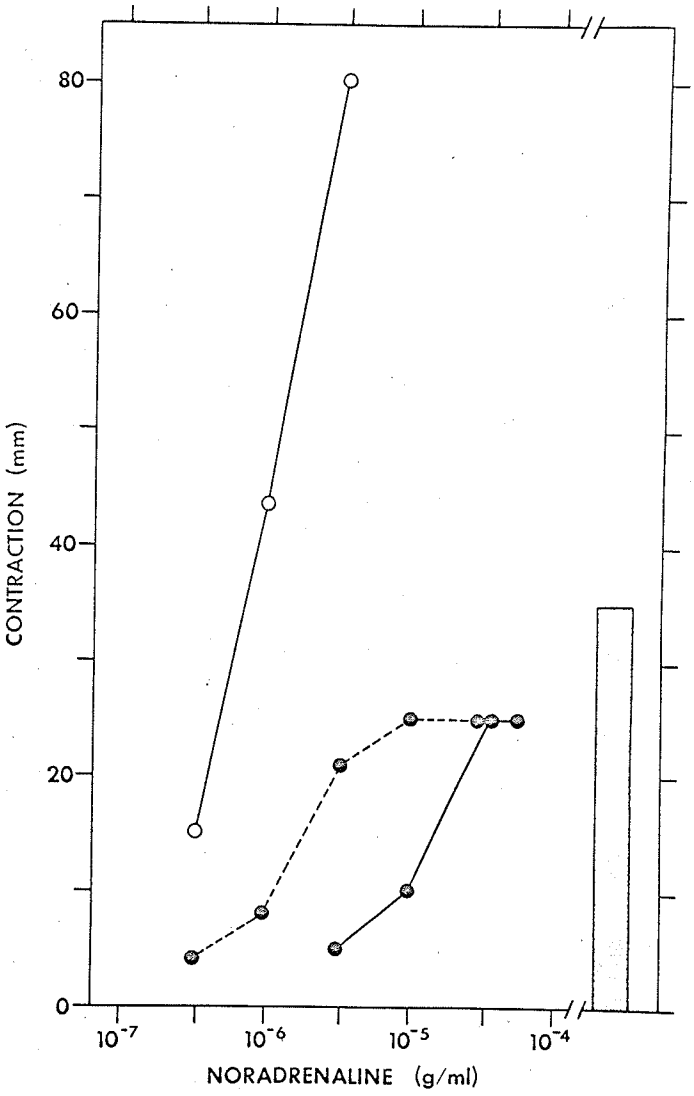

Fig. 20 EFFECT OF COCAINE ON THE DOSE-RESPONSE CURVE TO NORADRENALINE IN A CAT SPLEEN STRIP AFTER REMOVAL OF SPARE RECEPTORS.

Normal control responses O—O ; after phenoxybenzamine (5×10^{-8} g/ml) ●—● ; cocaine (3×10^{-6} g/ml) after phenoxybenzamine ●---●. The vertical column represents the contraction to 100 mM potassium after phenoxybenzamine.

after phenoxybenzamine. However, cocaine increased the responses to lower doses in 3 of the 4 experiments where these were tested (Fig. 20). Responses to all doses of noradrenaline in the 4 strips were not appreciably changed by cocaine. It was conceivable that the phenoxybenzamine had reduced the contractility of the tissue by some effect unrelated to its block of alpha receptors, so that the maximum response to noradrenaline was now the maximum of which the muscle was capable. The response to 100 mM potassium was therefore tested at the end of each experiment; in all four the potassium responses were greater than those for the noradrenaline (Fig. 20).

b. Isoproterenol

i. The partial agonist properties of isoproterenol

Testing the maximum response to a full agonist after phenoxybenzamine provides one method of eliminating the complication of spare receptors. A simpler way would be to test the maximum response to a partial agonist. Isoproterenol has been resported by Bickerton (1963) to be a partial agonist on alpha receptors in the cat spleen. In preliminary experiments, we found that the maximum response with isoproterenol, which required doses from 5×10^{-4} to 10^{-3} g/ml, was always less than with adrenaline. In order to confirm that isoproterenol was a partial agonist, we did an experiment with 4 spleen strips in which the cumulative response to graded doses were determined before and in the presence of constant doses of noradrenaline (10^{-7}, 5×10^{-7}, 5×10^{-6} & 10^{-5} g/ml). This procedure is based on the experiments of Ariens et al.(1956)

who showed that a partial agonist will act as an antagonist to a full agonist acting on the same receptors. When graded dose-response curves to a partial agonist are tested in the presence of fixed concentrations of a full agonist, higher doses of partial agonist will displace successively more full agonist until the receptors are totally occupied by partial agonist. The contraction under these conditions will not exceed the maximum for the partial agonist when tested alone. If the response to full agonist is initially low, then graded additions of partial agonist will cause an increased contraction to its own maximum. However, if a dose of full agonist is used which causes a contraction larger than the partial agonist maximum, graded additions of partial agonist will reduce the contraction as it approaches the partial agonist maximum.

The results are shown in Figure 21. In 2 strips noradrenaline caused a contraction which was less than the control isoproterenol maximum, and this was increased in one strip (A) by isoproterenol, and not appreciably changed in the other (B). In the other 2 strips, (C & D), noradrenaline caused a contraction which was greater than the maximum to isoproterenol. In one of these (D) addition of isoproterenol caused an initial increase in the contraction and a progressive inhibition; the other (C) showed only a decrease in contraction to isoproterenol. Since none of the responses in the presence of noradrenaline was ever increased by large doses of isoproterenol over its own control maximum, these results confirm that isoproterenol is a partial agonist on alpha receptors in the cat spleen.

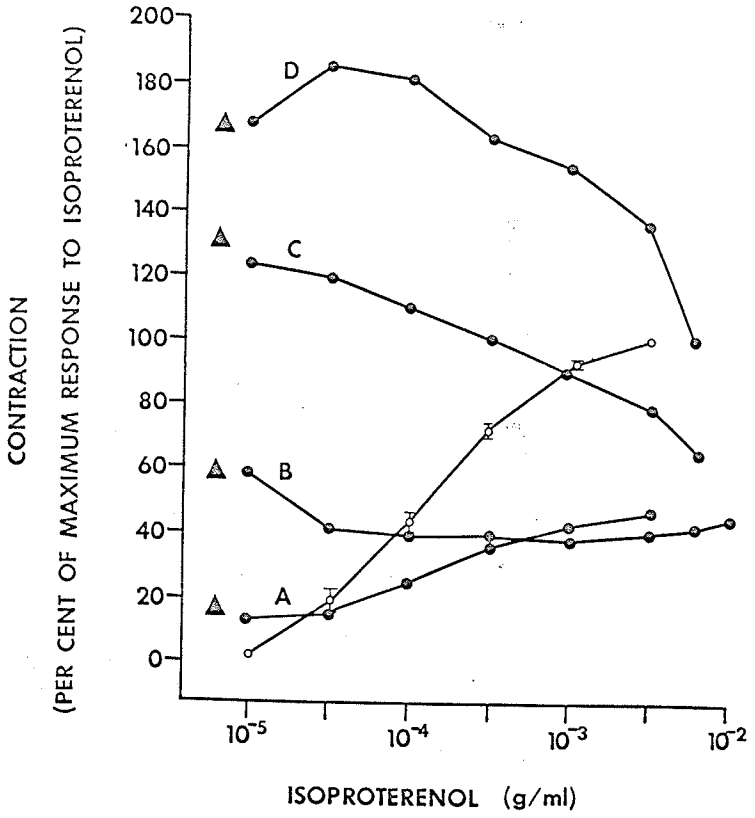

Fig. 21 A TEST FOR PARTIAL AGONIST PROPERTIES IN ISOPROTERENOL.

Ordinate: per cent of maximum contraction to isoproterenol. Abcissa: log dose of isoproterenol. Control curve (O—O) with standard errors is an average calculated from 4 strips. Curves A-D (●—●) show the effect in the individual strips of graded doses of isoproterenol in the presence of constant doses of noradrenaline (10^{-7}, 5×10^{-7}, $5 \times 10^{-6} + 10^{-5}$ g/ml) which caused the initial responses indicated along the ordinate (▲). The contractions of each strip are expressed as a percentage of the maximum control response for the same strip.

ii. The effect of cocaine on isoproterenol maximum

If cocaine increases the efficacy of the drug-receptor complex, then the maximum response to a partial agonist, such as isoproterenol, should be increased after cocaine. Normal spleen strips were contracted with 10^{-3} g/ml of isoproterenol. After 3 to 4 hours, during which the tissues were washed at 15 minute intervals, the same dose of isoproterenol was added again. In one group of 7 strips from 7 spleens, cocaine was added 5 minutes before the second response and remained in the bath during contraction. Four other strips from 4 spleens received no cocaine and served as controls for time. The results, summarized in Table 9, were examined by paired data analysis. The average difference between the first and second responses in the time controls was 15 ± 2.4 mm, an increase of 60.9 ± 4.5 per cent over the first response. This increase was statistically significant ($P < .001$). A small average increase in the cocaine treated group, 4.3 ± 3.7 mm, was not statistically significant.

It has been repeatedly shown that many sympathomimetic amines exert part of their effect by releasing noradrenaline from tissue stores (e.g. Fleckenstein & Burn, 1953; Trendelenburg, Muskus, Fleming & Gomez, 1962). The large increase in contraction seen with time in the control strips suggested to us that release of noradrenaline by large doses of isoproterenol might be complicating the response. We therefore repeated these experiments in spleen strips which had been depleted of their noradrenaline by reserpine (1 mg/kg) given to the cats 16 to 24 hours before the experiment.

TABLE 9

THE EFFECT OF COCAINE ON THE MAXIMUM RESPONSE TO ISOPROTERENOL IN ISOLATED SPLEEN STRIPS FROM NORMAL CATS

The difference between first and second responses of spleen strips to 10^{-3} g/ml of isoproterenol is expressed as per cent of the first response. The effect of cocaine (10^{-5}g/ml) added 5 minutes before the second response is compared with the effect of time.

Time				Cocaine			
Response (mm)		Difference		Response (mm)		Difference	
First	Second	mm	%	First	Second	mm	%
25	43	18	72.0	60	74	14	23.3
22	43	18	54.6	19	27	8	42.1
19	29	10	52.6	37	44	7	18.9
31	51	20	64.5	65	65	0	0
				33	18	-15	-45.5
				66	81	15	22.7
				55	52	-3	-5.4
	Mean	15.0	60.9		Mean	4.3	8.0
	±S.E.	2.4	4.5		± S.E.	3.7	51.4

Eight strips from 8 spleens served as time controls, and 12 other strips from 12 spleens received cocaine before the second response. The results are summarized in Table 10. The control strips showed a slight average decrease with time (3.9 mm) which was not statistically significant. With cocaine the second response showed a statistically significant increase of 13.3± 3.7 mm, 60.5 per cent, over the first response.

Without further analysis, these data might be interpreted as indicating that cocaine causes an increase in the maximum response to isoproterenol which is seen only when the indirect action of isoproterenol is abolished by depleting the noradrenaline stores. A tacit assumption behind this paired data analysis is that the initial responses measured are representative samples of a common population. Figure 22 represents in bar-graph form the first and second responses obtained in each of the four test groups. The initial responses fall into dissimilar groupings. For the normal spleens the average value of 47.9 ± 6.9 mm for the initial responses preceding cocaine (column B_1) is much greater than the average initial responses of 24.3 ± 2.6 mm in the time control group (column A_1). These values are significantly different ($P < .05$). Comparison of initial responses in the reserpine-treated group shows a wide difference in the opposite direction. Thus the initial responses before cocaine averaged 29.5 ± 4.5 mm (column D_1), and those which served as time controls (column C_1) were much larger at 63.6 ± 11.2 mm. There is a highly significant difference between these groups also ($P < .001$). The greatest differences between the first and second

TABLE 10

THE EFFECT OF COCAINE ON THE MAXIMUM RESPONSE TO ISOPROTERENOL IN ISOLATED SPLEEN STRIPS FROM CATS TREATED WITH RESERPINE

The difference between first and second responses of spleen strips to isoproterenol (10^{-3}g/ml) is expressed as per cent of the first response. The effect of cocaine (10^{-5}g/ml) added 5 minutes before the second response is compared with the effect of time. All spleens were taken from cats which had been given reserpine (1 mg/kg) 16 - 24 hours earlier.

Time				Cocaine			
Response (mm)		Difference		Response (mm)		Difference	
First	Second	mm	%	First	Second	mm	%
30	30	0	0	33	72	39	118.2
43	24	-19	-44.2	14	32	18	128.6
19	16	-3	-15.8	14	36	22	157.1
59	39	-20	-33.9	63	69	6	9.5
67	59	-8	-11.9	18	30	12	66.7
103	105	2	1.9	18	31	13	72.2
92	122	30	32.6	42	76	34	81.0
96	83	-13	-13.5	16	17	1	6.3
				37	37	0	0
				32	49	17	53.1
				20	34	14	70.0
				47	30	-17	-36.2
	Mean	-3.9	-10.6		Mean	13.3	60.5
	±S.E.	21.5	51.5		±S.E.	3.7	14.1

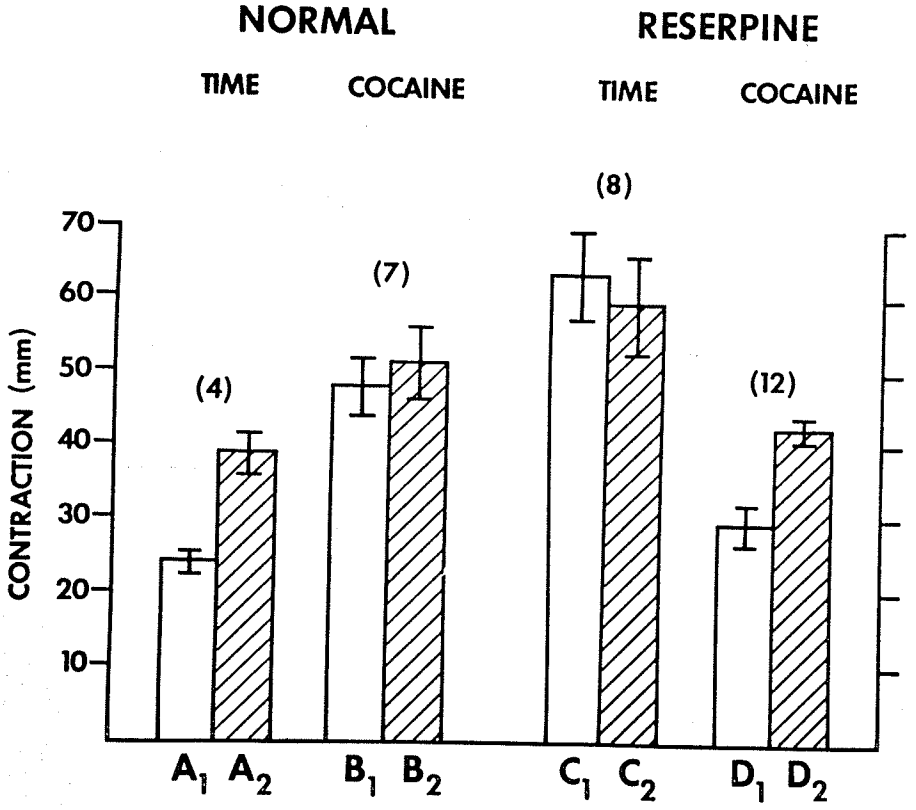

Fig. 22 AVERAGE FIRST AND SECOND RESPONSES TO A LARGE DOSE OF ISOPROTERENOL IN CAT SPLEEN STRIPS.

Groups A & B: normal spleen strips. Groups C & D: spleen strips from reserpine treated cats (1 mg/kg 16-24 hr before experiment). Open columns, first response; closed columns, second response to isoproterenol (10^{-3} g/ml). Vertical lines show standard errors. Groups B & D received cocaine (10^{-5} g/ml) 5 minutes before the second response was determined. Numbers in parentheses show the number of strips in each group.

Statistical comparisons

A_1 v A_2: $P < .05$ A_1 v B_1: $P < .001$

D_1 v D_2: $P < .001$ C_1 v D_1: $P < .001$

responses occurred where the initial responses were the lowest (groups A & D). Therefore, the increase seen after cocaine in the reserpine treated group may well have been related to the low initial response rather than to any effect of cocaine.

2. The effect of cocaine on steady-state maximum responses

Because of the variability inherent in biological preparations, duplicate tests within the same tissue often give different values. This has been clearly demonstrated in the experiments on isoproterenol maxima just described. In the experiments to follow, this type of variability was eliminated by testing only one maximum response to the agonist. The effect of the sensitizing drug was tested only after a steady-state was reached.

a. Noradrenaline

Full maximum

Fourteen spleen strips from 7 cats were maximally contracted with noradrenaline (5×10^{-5} to 10^{-4} g/ml) to reach a steady-state contraction. Cocaine (10^{-6} or 10^{-5} g/ml) was added during the steady-state, but never appreciably changed the amplitude of the response (Fig. 23a).

In several of these experiments phenoxybenzamine was added after the effect of cocaine had been determined. This constituted a separate experiment on the decay of steady-state contractions, described earlier on page 73 , and did not influence the effect of cocaine on maximum responses.

Reduced maximum

In 2 strips from the same spleen, phenoxybenzamine (10^{-6} g/ml) was added during a steady-state response to 6×10^{-5} g/ml

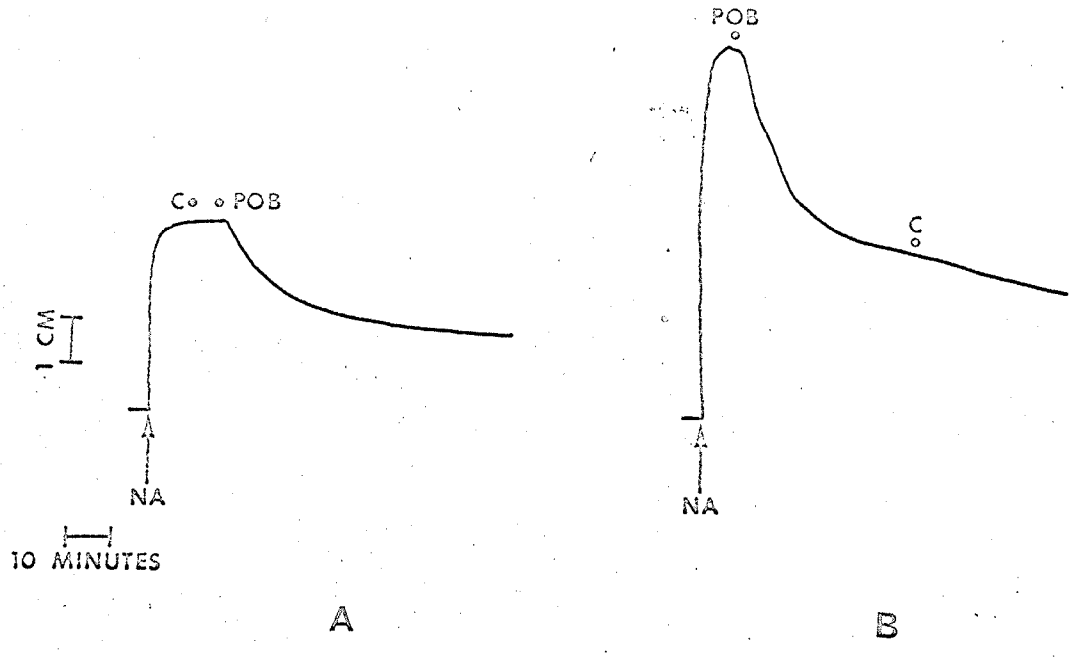

Fig. 23 THE EFFECT OF COCAINE ON THE STEADY-STATE MAXIMUM RESPONSE TO NORADRENALINE.

A: cocaine (10^{-6} g/ml) was added at C during the steady-state contraction to noradrenaline (5×10^{-5} g/ml). [Phenoxybenzamine (POB) added later was part of a separate experiment.] B: Phenoxybenzamine (10^{-6} g/ml) was added during the steady-state contraction to noradrenaline (6×10^{-5} g/ml). Cocaine (10^{-5} g/ml) was added at the new steady-state.

of noradrenaline. This caused a fall in the response which gradually approached a new level, presumably due to a reduction of available receptors by phenoxybenzamine below the number necessary to cause a full maximum response. Cocaine (10^{-5} g/ml) added during this reduced maximum response caused no detectable change in amplitude (Fig. 23b).

These experiments indicate that both the full maximum contraction to noradrenaline and the reduced maximum after removal of a large portion of receptors are not increased by cocaine.

b. Isoproterenol

Full maximum

Five spleen strips from 4 spleens were contracted maximally by isoproterenol (10^{-3} g/ml). Two of the spleens were from cats given 1 mg/kg of reserpine 16 to 24 hours earlier. After the response became constant, cocaine (3×10^{-5} g/ml) was added but did not increase the contraction in any of the strips (Fig. 24a).

Reduced maximum

Five other strips from the same four spleens were given phenoxybenzamine (3×10^{-11} to 10^{-9} g/ml) during the steady-state contraction. The responses were reduced by about 50% after all but the highest dose, which reduced the response to about 10% of its original value. When the new level was approached, 3×10^{-5} g/ml of cocaine was added. No change in contraction occurred (Fig. 24b).

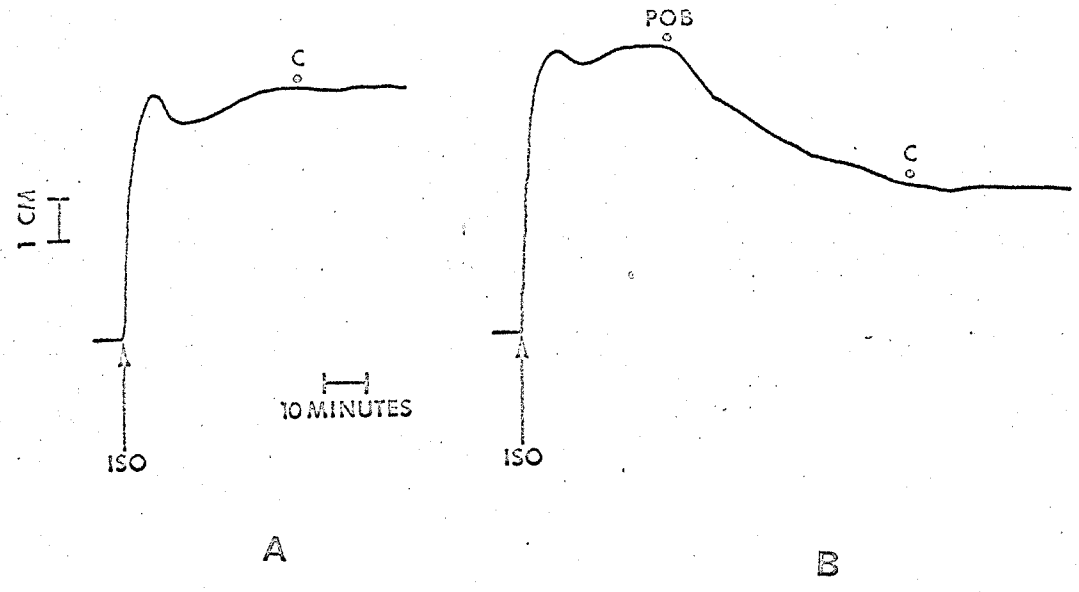

Fig. 24 THE EFFECT OF COCAINE ON THE STEADY-STATE MAXIMUM RESPONSE TO ISOPROTERENOL.

A: cocaine (3×10^{-5} g/ml) was added at C during the steady-state contraction to isoproterenol (10^{-3} g/ml). B: Phenoxybenzamine (POB) (10^{-10} g/ml) was added during the steady-state contraction. Cocaine (3×10^{-5} g/ml) was added at the new steady-state.

B. THE RESPONSE TO CALCIUM RESTORATION IN THE PRESENCE OF ADRENALINE

It is well known that the calcium ion is an essential coupling factor in the contraction of smooth muscle to all forms of stimuli (Daniel, Sedhev & Robinson, 1962; Waugh, 1962; Hinke, 1965). A study of its utilization in contraction should therefore provide an index of post-receptor events in the stimulus-response sequence.

We have designed experiments on calcium utilization to minimize the effects of variability in pre-receptor and receptor events. Strips depleted of calcium were stimulated with a large dose of noradrenaline (5×10^{-5} g/ml) which would saturate the receptors and uptake sites. We presumed that all the receptors were occupied by agonist, so that the height of the contraction caused by graded calcium restoration would be an index of the ease with which calcium ions reached and activated the contractile mechanism. The effects of denervation and cocaine (10^{-6} g/ml) were studied on the response to calcium restoration. Cocaine was studied both in normal spleen strips and strips from reserpine treated cats.

To deplete the preparation of calcium as fully as possible, large doses of noradrenaline and histamine (1 or 5×10^{-5} g/ml) were given alternately over a 4 hour period during which the tissue was bathed in zero-calcium medium (Fig. 25). Successive responses to noradrenaline at first declined, but reached a minimum level which was not reduced by additional doses. In contrast, contractions to histamine in zero-calcium medium were always much smaller than those to noradrenaline, and usually decreased to

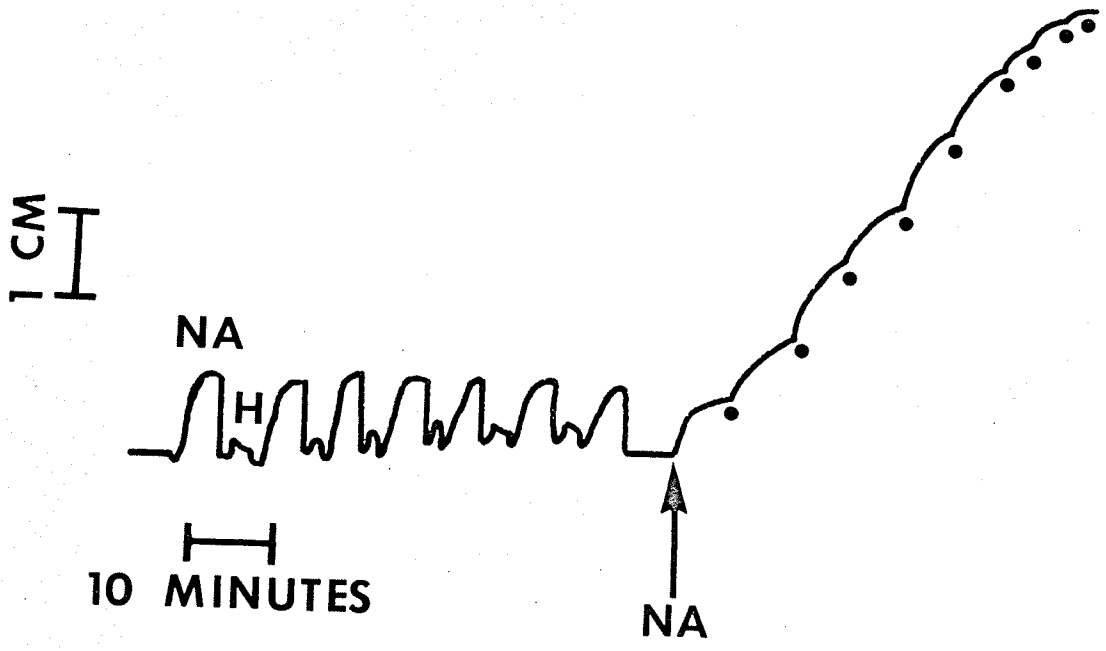

Fig. 25 THE EFFECT OF RESTORATION OF CALCIUM ON SPLEEN STRIP CONTRACTION DUE TO A SUPRAMAXIMAL DOSE OF NORADRENALINE IN ZERO-CALCIUM MEDIUM.

Contractions to 10^{-5} g/ml noradrenaline (NA) and 10^{-5} g/ml histamine (H) were alternated in zero-calcium bathing fluid. At the arrow, NA (10^{-4} g/ml) was added and left in the bath. At each dot calcium was added, the addition being made only after the previous contraction no longer increased. The final concentrations of calcium after each successive addition were 0.1, 0.2, 0.5, 1.0, 2.0, 5.0, 10.0 and 20.0% of the concentration in Krebs-Henseleit solution (2.5 mM).

barely detectable levels during the calcium-free test period.

Thus the dose of noradrenaline used in these experiments always caused a response in the absence of calcium. Table 11 shows the values for these contractions expressed as per cent of the maximum response after calcium was added. The differences between the average values for each treatment are not statistically significant.

The strips responded with graded increases in contraction to the cumulative additions of calcium (Fig. 25). Table 12 shows the calcium concentrations to cause 50 per cent of the maximum contraction in normal and treated spleens. This was always much less than the concentration in standard Krebs-Henseleit solution, but was highly variable, ranging from 0.14 to 6.2 per cent. There were no statistically significant differences in the average calcium concentrations causing a 50 per cent response. Comparison of the normal controls and reserpine treated controls ($1.65 \pm .63$ v 0.74 ± 0.18) yielded the lowest value for p ($> .2$). None of the sensitizing procedures could therefore be coupled with an effect on utilization of calcium in the bathing fluid for contraction.

TABLE 11

RESIDUAL CONTRACTIONS TO NORADRENALINE ($1\text{-}5 \times 10^{-5}$ g/ml) NOT ABOLISHED BY BATHING IN ZERO-CALCIUM MEDIUM

Spleen strips from cats treated as indicated were bathed in zero-calcium Krebs-Henseleit medium and contracted repeatedly with noradrenaline until constant responses were obtained. The value of the last response is expressed in the table as per cent of the maximum response to noradrenaline after calcium was replaced. For reserpine treatment 1 mg/kg was given 16 - 24 hours before the experiments. When more than one determination was made on a single spleen, the average value is shown in the table.

Denervation		Cocaine			
		Normal		Reserpine-treated	
Control %	Denervated %	Control %	Cocaine %	Control %	Cocaine %
19.5	20.5	25.5	20.0	23.0	30.5
23.0	15.0	18.0	13.5	18.5	19.0
6.0	7.5	12.0	12.0	15.5	13.0
		3.0	2.0	5.0	3.0
Mean 16.2	14.3	14.6	11.9	15.5	16.4
±S.E. 5.18	3.99	4.75	3.72	3.82	5.75

TABLE 12

CALCIUM CONCENTRATION IN BATHING FLUID YIELDING 50% OF THE MAXIMUM RESPONSE IN SPLEEN STRIPS ACTIVATED BY NORADRENALINE (5×10^{-5} g/ml)

Spleen strips treated as shown in the table were bathed in zero-calcium medium until the response to repeated doses of noradrenaline showed no further reduction. Noradrenaline (5×10^{-5} g/ml) was added and left in the bath while the calcium concentration was increased in graded amounts. Values in the table are calcium concentrations calculated for the response at 50% of maximum. When more than one determination was made on a spleen, the average of the values is shown in the table. For reserpine treatment 1 mg/kg was given 16 - 24 hours before the experiment. Calcium concentration is given as per cent of the Krebs-Henseleit concentration (2.5 mM).

Denervation		Cocaine			
		Normal		Reserpine-treated	
Control	Denervated	Control	Cocaine	Control	Cocaine
4.30	6.21	2.70	5.35	1.06	1.33
0.55	0.49	0.34	0.51	0.73	0.81
0.14	0.47	2.75	1.40	0.25	0.31
		0.79	0.50	0.92	1.65
Mean 1.66	2.39	1.65	1.94	0.74	1.03
±S.E. 1.33	1.91	0.63	1.15	0.18	0.29

DISCUSSION

PART ONE

RESULTS BEARING ON THE UPTAKE HYPOTHESIS

A. COCAINE PROTECTION STUDIES

These experiments provide strong evidence that the effect of cocaine on catecholamine response is independent of the effect of cocaine on catecholamine uptake. The reasoning may be summarized thus: (a) competitive antagonism of potentiation indicates that cocaine acts to influence receptor occupancy by catecholamine, (b) substances with known affinity for the uptake mechanism provide little or no protection against cocaine potentiation, and (c) an agent which affords striking protection in our experiments (phentolamine) has been demonstrated by others to have little or no effect on catecholamine uptake.

Cocaine Competition and Potentiation

The evidence for a competitive interaction between cocaine and sympathomimetics (page 21) provides a suitable basis to test the uptake hypothesis: competitive exclusion of cocaine from sites of uptake should protect against the supersensitivity it causes if this is a consequence of reduced uptake. While it was obvious that drugs known to interact with the uptake process, such as noradrenaline and tyramine, should be tested as protecting drugs, we wanted to include other agents known either to initiate or interfere with responses in the cat spleen.

Noradrenaline may have afforded some protection against cocaine potentiation, but the effect was not dramatic, since cocaine still caused a substantial increase in the noradrenaline response. Tyramine, like noradrenaline, has a high affinity for specific catecholamine uptake sites (Burgen & Iversen, 1965). However, unlike noradrenaline, it has little affinity for alpha receptors.

It has been effectively shown that the uptake of tyramine by sympathetic nerve endings is competitively inhibited by cocaine, since the antagonism is surmountable by increasing the dose of tyramine (Lockett & Eakins, 1960; Trendelenburg, 1961; Furchgott et al., 1963). If block of the uptake process by cocaine were the cause of supersensitivity, then tyramine, by protecting uptake sites from combination with cocaine, should prevent the supersensitivity. Its complete failure to protect against supersensitivity does not support a functional relation between uptake and potentiation.

Our results with phentolamine provide the strongest evidence against the uptake hypothesis. This drug has no significant affinity for the uptake mechanism (Hertting, Axelrod & Patrick, 1962; Dengler, 1965), yet provided complete protection against the sensitizing action of cocaine under carefully controlled conditions. Thus the possible effect of the alpha receptor block by phentolamine was controlled by measuring this effect on a separate tissue. Comparisons of sensitivity changes after cocaine and cocaine plus phentolamine were not made until noradrenaline responses in the phentolamine control had returned after washout of the antagonist to the original level obtained before phentolamine. Since the times of addition and removal of phentolamine were the same for both tissues receiving it in any experiment, alpha receptor antagonism was unlikely in either tissue when sensitivities were compared. This might not be true if cocaine prolonged receptor occupancy by phentolamine. For this reason, the experiments described on page 60 were done. If cocaine decreased the dissociation of

phentolamine from alpha receptors, then this should be seen as an increase in the time required for noradrenaline to overcome a given level of block after phentolamine has been removed from the bathing fluid. Since there was no significant difference in the time required for a noradrenaline-induced contraction to reach 50 per cent of its eventual maximum level after phentolamine alone or after phentolamine in the presence of cocaine, cocaine does not appear to alter the duration of phentolamine block of alpha receptors.

Thus the phentolamine antagonism of cocaine potentiation is not a deception due to residual alpha receptor block. We have made no measurements in our preparation to test directly the possibility that cocaine might interfere with catecholamine uptake. However, reports from two separate laboratories have indicated that the action of phentolamine in this respect is not significant at doses comparable to ours. Hertting, Axelrod and Whitby (1961) showed that phentolamine (5 mg/kg) had no effect on the uptake of subsequently injected H^3- noradrenaline by rat heart and spleen. Dengler's experiments on cat spleen slices (1965) showed that phentolamine (5.6×10^{-7} g/ml) caused a slight (14%) depression of H^3- noradrenaline uptake. By contrast, 3.4×10^{-7} g/ml of cocaine (less than a hundredth of the dose used in our protection experiments) caused 80-100% per cent inhibition of uptake. In view of these data, it does not seem likely that block of uptake by our relatively large dose of cocaine (10^{-5} g/ml) would be effectively antagonized by the dose of phentolamine used (3×10^{-6} g/ml). Assuming this, we must seek another site of action

for cocaine to explain its sensitizing effect. This site should be competitively affected by phentolamine, cocaine, and possibly noradrenaline. The combination of cocaine at this site should be unaffected by tyramine, isoproterenol, histamine and acetylcholine. Although our experiments provide no data bearing directly on the nature of the site for cocaine sensitization, it is useful to speculate on the possibilities the data permit.

Although cocaine can reduce amine oxidase activity, very high concentrations are required (Philpot, 1940), and other more potent inhibitors are known which do not potentiate noradrenaline (Griessmer et al., 1953). Cocaine is therefore unlikely to exert its physiological effects in this way. The only other enzyme of known significance in the activation of catecholamines is catechol O-methyltransferase (Axelrod, 1957). O-methylation is responsible for nearly 50 per cent of the early disappearance of injected noradrenaline, and the remaining 50 per cent is bound in the tissues (Whitby, Axelrod & Weil-Malherbe, 1961). Hertting & Axelrod (1961) showed that noradrenaline released by nerve stimulation meets a similar fate. Drugs which inhibit this enzyme do enhance responses to noradrenaline (Wylie, Archer & Arnold, 1961). Cocaine, however, like other drugs which prevent entry of noradrenaline into adrenergic neurones, actually increases the proportion of O-methylated metabolites after the administration of noradrenaline (Axelrod, 1966).

At what site, then might cocaine act to increase receptor occupancy by catecholamine? Current interest in the problem of catecholamine potentiation has centered on altered mechanisms of

disposal which would increase the concentration of drug in the biophase (D_b in Fig. 1). Receptor occupancy could also be increased in a different way, namely by increasing the affinity of the receptors for the drug (k_1/k_2 in Fig. 1). This possibility was alluded to by Clark (1937), but seems to have received little attention by recent investigators. In our experiments, the drug which provided greatest protection (phentolamine) has a high affinity for the alpha receptor. The only other drug that appeared to cause some reduction of potentiation (noradrenaline) also has a high affinity for alpha receptors. This might suggest that cocaine combines with the alpha receptor itself to cause supersensitivity. However, this is difficult to imagine, since direct combination with the alpha receptors would make cocaine an antagonist, which it clearly is not. Another possibility is that cocaine may influence the alpha receptor by combining with a different site nearby. Molecular interactions between the drug and protein molecules could then induce conformational changes in the alpha receptor resulting in increased affinity for drugs which combine with it. Although there is no evidence for this hypothesis in cellular drug-receptor systems, it represents a close analogy with current theory regarding allosteric interactions in enzyme kinetics (e.g. Koshland, 1963, 1964; Monod, Changeaux & Jacob, 1963). It has been suggested that the active site of an enzyme may be flexible, and that its ability to bind substrate can easily be modified by both activators and inhibitors which alter the conformation of groupings critical to the binding process (Koshland, 1963). It has further been suggested that activators and inhibitors may

mutually interact, and that the presence of one may have stabilizing effects which decrease the combination of the other with the protein. (Koshland, 1964). It is not difficult to adapt this hypothesis to explain our results with phentolamine and cocaine. Thus cocaine could be an allosteric activator of alpha receptors. Phentolamine could so change the conformation of the protein around the alpha receptor that it (a) stabilizes the receptor and prevents the conformational change due to cocaine resulting in affinity increase, and (b) prevents or reduces the combination of cocaine with its site so that potentiation does not occur when the phentolamine is washed out of the tissue. The difference between the ability of noradrenaline and phentolamine to protect could be due to the fact that noradrenaline, being an agonist, does not increase the stability of the protein near the receptors and therefore does not affect the binding of cocaine. The poorer affinity of noradrenaline for alpha receptors, relative to phentolamine, could also explain the difference between their effects in the doses used.

A point emphasized by Monod et al.(1963) is that "the primary reason for considering allosteric proteins as essential and characteristic constituents of biochemical control systems is their capacity to respond immediately and reversibly to specific chemical signals, which may be totally unrelated to their substrates, coenzymes, or products" (Authors' italics). Cocaine bears no close resemblance to structures which combine either with alpha receptors or the catecholamine uptake sites. In this sense, it is an excellent candidate for the hypothesis of allosteric interaction at the

alpha receptor. The information which has led to the theory of allosteric interaction for enzymes has been based on kinetic studies. Although the cellular drug-receptor system is much more complex than a simple enzyme-substrate-product system, it nonetheless might be profitable to study the kinetics of single responses to varying concentrations of agonist ("substrate"). Changes in the velocity of response due to activators and inhibitors could provide analogous information to that obtained for enzyme systems.

It should be noted that the application of allosteric concepts to receptor pharmacology is only an attempt to reconcile the data we have obtained. However, our knowledge of mechanisms is still so vague that it is not unreasonable to challenge the classical concept of competitive isosteric drug interaction just as Koshland, Monod, and others have challenged the template theory of enzyme action.

B. POTENTIATION OF STEADY-STATE RESPONSES BY COCAINE

Noradrenaline

As discussed in the Introduction (page 24 & Fig.1), it is difficult to conceive how uptake can limit the biophase concentration (D_b, Fig. 1) in vitro. The relatively large reservoir in the bathing fluid (D_a) will not be appreciably changed by the uptake which occurs (D_s), and since the biophase is in equilibrium with the bathing fluid, the final concentration of drug in the tissue will be similarly unchanged. Thus blocking the uptake mechanism before the agonist is added would not be expected to increase the biophase concentration. In vitro potentiation of noradrenaline

responses by cocaine is therefore not readily explained by inhibition of uptake.

This point is further supported by the experiments in which cocaine was added to tissues during the steady-state of submaximal noradrenaline responses. The biophase concentration of drug would then have achieved equilibrium with the uptake mechanism. Cocaine would then increase D_b only by increasing k_a or k_b, or by promoting release from D_s. Dengler (1965) has studied the effect of drugs on the efflux of noradrenaline from slices of cat spleen. After a 30 minute incubation with labelled noradrenaline, 5×10^{-5}M concentrations of drugs were added and their effect on noradrenaline efflux determined. Of all the drugs tested, cocaine was the most effective inhibitor of efflux at both 20 and 60 minutes after exposure. Cocaine never stimulated efflux, as did tyramine, reserpine, and chlorpromazine. Dengler's results indicate that cocaine would not promote release from uptake sites at concentrations normally used to cause potentiation of catecholamines.

The foregoing discussion has centered around the assumption that cocaine increases receptor occupancy by increasing the concentration of drug at the receptors (D_b). An alternative to this is the possibility that cocaine acts on the receptors to increase k_1/k_2, i.e. to increase the affinity of the receptors. Either this, or an increase in k_a/k_b (biophase permeability) seem to be equally plausible explanations for these results, and far easier to accept than a block of uptake.

A change in k_a/k_b would alter the distribution of drug between aqueous phase and biophase. This should be detectable as

a change in total biophase concentration when cocaine is added during the steady-state. Measurement of tissue noradrenaline concentration in a parallel experiment should therefore enable a distinction between increased permeability on the one hand, and increased affinity on the other.

Isoproterenol

In contrast to noradrenaline, isoproterenol is not concentrated by sympathetically innervated tissues (Hertting, 1964; Hardman, Mayer & Clark, 1965), and has virtually no affinity for the uptake mechanism, as judged by its inability to inhibit noradrenaline uptake in several tissues (Burgen & Iversen, 1965). Butterworth (1963) showed that large doses of isoproterenol cause a pressor response in anesthetized cats, and that cocaine failed to potentiate this response. These reports have been regarded as compatible with the uptake hypothesis, which would predict failure of cocaine to potentiate an agonist with low affinity for uptake sites.

However, our results clearly showed that cocaine potentiates steady-state submaximum contractions to isoproterenol in cat spleen strips. Isoproterenol uptake has not been studied in this tissue, and it is conceivable that in this respect it differs from the other tissues in which isoproterenol uptake has been studied. Even if this were possible, the same arguments which were raised for noradrenaline in the previous section make it unlikely that reduced uptake of isoproterenol is the basis of its potentiation by cocaine.

The potentiation of noradrenaline and isoproterenol in the cat spleen by cocaine further emphasize the need to seek an

alternative explanation for cocaine supersensitivity, already indicated by our results with phentolamine in the cocaine protection experiments (page 58).

PART TWO

EXPERIMENTS WITH ALPHA RECEPTOR ANTAGONISTS

A. SUPERSENSITIVITY OF THE DENERVATED SPLEEN

Our results with denervated spleens confirm and extend the earlier observations of Burn & Rand (1959) who studied the volume changes of the whole cat spleen *in situ* in response to noradrenaline and reported this response to be enhanced after sympathetic denervation. We also have observed supersensitivity of the denervated cat spleen, but our findings were made on spleen strips contracted *in vitro* with noradrenaline. In the Introduction, reasons were stated which made it unlikely that changes in uptake could alter the equilibrium biophase concentration of noradrenaline *in vitro* (page 24). Since we have observed marked supersensitivity of denervated spleen strips *in vitro* some mechanism other than loss of uptake is probably involved.

B. SUPERSENSITIVITY AFTER RESERPINE

Trendelenburg and co-workers have repeatedly stated that in their experiments supersensitivity does not follow short-term reserpine treatment (Fleming, 1963; Trendelenburg, 1965; Trendelenburg & Pfeffer, 1964). Our results are at variance with their observations. In all our experiments supersensitivity to noradrenaline appeared 16 to 24 hours after treatment with 1mg/kg reserpine. Others have reported similar changes. Innes (1960) found supersensitivity in the cat nictitating membrane within 5 minutes of giving reserpine; the sensitivity continued to increase during the 16 hour recording period. Although the initial responses may have been influenced by catecholamines released by reserpine (Trendelenburg, 1963a)this factor should be eliminated long before the 16 hour recordings. Brutsaert

(1964) reported supersensitivity in the isolated or perfused cat pulmonary vascular bed within 16 to 24 hours of 2.4 to 2.6 mg/kg reserpine. Hudgins and Fleming (1966) observed supersensitivity to noradrenaline in the rabbit aorta 24 hours after 1 mg/kg of reserpine. Differing experimental conditions may underlie the discrepancy between Trendelenburg's findings and our own. However, we believe that our positive findings are convincing, and that the apparent inability of reserpine to cause supersensitivity under certain conditions should be carefully re-examined.

C. PHENTOLAMINE ANTAGONISM

Phentolamine block of noradrenaline was not enhanced by denervation or reserpine treatment in any of our experiments with pA_{10} measurements. There is strong evidence which indicates that phentolamine has negligible effect on the uptake mechanism (page 105). In view of this, our data might appear to be in harmony with the uptake hypothesis; if the concentration of the drug reaching the receptor is not normally influenced by the catecholamine uptake mechanism, then removal of uptake should not enhance its antagonism.

However, the possibility that receptor affinity is increased in supersensitivity would also be consistent with our observations. Phentolamine is a reversible competitive antagonist. Its antagonistic ability, measured in our experiments as inhibition of the noradrenaline response, is always a resultant of a complex equilibrium between agonist, antagonist, and the alpha receptors. If loss of uptake sites significantly increased the active noradrenaline concentration at the receptors, but had no effect on

the free phentolamine concentration, decreased antagonism would be predicted. The very fact that phentolamine antagonism was unchanged in tissues supersensitive to noradrenaline is strong evidence that phentolamine activity correspondingly increased. In view of the relative resistance of phentolamine to other processes which normally inactivate the alpha receptor agonists, it is most likely that this increased antagonism represents an increase in receptor affinity. Thus, our data with phentolamine, contrary to supporting the uptake hypothesis of supersensitivity, actually provide indirect evidence of an increase in receptor affinity.

D. PHENOXYBENZAMINE ANTAGONISM

We have shown that phenoxybenzamine block of alpha receptors is increased in spleen strips made supersensitive by cocaine or chronic denervation. These results may be interpreted as due to an increased receptor affinity for phenoxybenzamine. If phenoxybenzamine were appreciably taken up by nerve endings in normal tissues, these results might also be explained by the uptake theory. Although several reports have indicated that tissue catecholamines are reduced by phenoxybenzamine, the observation can often be explained by actions other than block of the tissue uptake mechanism.

Brown & Gillespie (1957) showed that noradrenaline in the venous effluent of the spleen after low frequency splenic nerve stimulation is increased by phenoxybenzamine. They attributed the increase to the effect of alpha receptor block and proposed that combination with receptors was necessary for the destruction of

released noradrenaline, Hertting, Axelrod & Whitby (1961) showed that after an infusion of H^3-noradrenaline tissue levels of the amine were lower and plasma levels higher in cats treated with phenoxybenzamine than in cats without phenoxybenzamine, and suggested that block of uptake by tissue stores might be the cause. Farrant, Harvey & Pennefather (1964) showed that the noradrenaline content of some rat and cat tissues was reduced by chronic phenoxybenzamine treatment. They also showed that a single large dose of phenoxybenzamine (6 mg/kg) reduced tissue uptake from a noradrenaline infusion in some cat tissues, while other tissues, notably uterus and duodenum, were unaffected. A single dose of phenoxybenzamine given before an infusion of noradrenaline decreased the noradrenaline content of the heart measured after the infusion. They concluded that phenoxybenzamine must act partly to block uptake of noradrenaline, since noradrenaline does not act on alpha receptors in the heart. Their data indicate that pre-infusion catecholamine levels were significantly lowered in some tissues (most notably spleen) by a single dose of phenoxybenzamine. This effect is most likely due to the release of stored catecholamine. The uptake process may therefore be operating normally after phenoxybenzamine and the decreased net accumulation of catecholamine from an infusion could be due to alteration in the storage mechanism and consequent rapid release of the accumulated catecholamine.

The net tissue level after an infusion could be reduced either by a decreased influx, or by an increased efflux. Hertting et al.(1962) showed that H^3-noradrenaline in the rat heart after an

infusion was decreased similarly by phenoxybenzamine, reserpine or guanethidine whether the drugs were given before or after the infusion. They concluded that these drugs must act by releasing or preventing binding of noradrenaline. In the same year, Weiner, Draskoczy & Burack showed that phenoxybenzamine did not prevent noradrenaline release by tyramine in adrenalectomized, anaesthetized dogs. Block of uptake by phenoxybenzamine would be expected to exert a cocaine-like inhibition of the action of tyramine. Costa et al.(1966) showed that the effect of phenoxybenzamine on H^3-noradrenaline uptake by the rat heart and colon was transient, whereas the loss due to nerve stimulation was increased long after uptake has returned to normal. Phenoxybenzamine increased the spontaneous efflux from the same preparation. Kirpekar & Cervoni (1963) showed that both phentolamine and phenoxybenzamine increased noradrenaline output with splenic nerve stimulation, although phentolamine had no effect on noradrenaline uptake. When the effect of phenoxybenzamine on adrenaline release by splanchnic nerve stimulation was studied, cocaine and phentolamine had no effect, but phenoxybenzamine increased the output at all frequencies.

The evidence currently available indicates that phenoxybenzamine has important actions to release noradrenaline, first suggested by Furchgott in 1959, and to prevent alpha receptor-dependent metabolism of released noradrenaline. There is no direct evidence to suggest that any of its effects are due to a block of catecholamine uptake sites which lead to storage.

Because of the non-equilibrium nature of the antagonism

at alpha receptors by phenoxybenzamine (Nickerson & Nomaguchi, 1951), measurement of the antagonism does not require simultaneous exposure to both agonist and antagonist. If the antagonist is added first for a limited time period, and then the excess which has not combined with tissue elements is removed, a relatively permanent block will be established by the remaining drug. This block does not occur instantly, but through a reactive iminium intermediate, formation of which is relatively slow (Nickerson & Gump, 1949; Nickerson, 1957). Part of the unreacted drug may be removed from the biophase by washing the tissue during the initial period after administration. The half-life of this disappearance will vary from 10 to 25 minutes, during which a residual nonequilibrium block is established which is not removed by further washing (Nickerson & Gump, 1949).

In our dose-ratio experiments with phenoxybenzamine, 30 minutes was allowed after adding the antagonist before testing noradrenaline responses. This was regarded as adequate time for development of a substantial component of irreversible receptor block. If phenoxybenzamine does not combine appreciably with the uptake mechanism, our results showing an increased block after denervation can be interpreted as an increased binding capacity for phenoxybenzamine by the alpha receptors.

The difference between these results and those with phentolamine, where no change in antagonism was seen, can be explained by the qualitative difference in their antagonism. With phentolamine, where a complex agonist-antagonist equilibrium is established, a

similar increase in affinity for both drugs would oppose any net change in the measured antagonism. On the other hand, irreversible phenoxybenzamine block was established before agonist was added. Once the block was established, the presence of agonist would not appreciably alter it, regardless of any increase in affinity. Increased affinity of the receptors for antagonist would thus be expected to be seen as a greater block of the agonist.

Phenoxybenzamine caused equal antagonism of noradrenaline in normal and in reserpine treated spleens. The lack of any difference cannot be attributed to any absence of supersensitivity, for noradrenaline responses were consistently greater in the reserpine treated tissues. Innes (1960) has shown that nictitating membrane supersensitivity after reserpine is qualitatively similar to that after decentralization, and different from that of denervation. Thus, as discussed on page 6 , reserpine causes nearly equal sensitization to most agonists in several smooth muscle preparations. If the sensitizing action of reserpine were at a post-receptor level, receptor occupancy by phenoxybenzamine would not be affected. We believe that reserpine does act at a post-receptor level, and will discuss the evidence for this opinion in a later section.

Our experiments indicate that phenoxybenzamine antagonism is increased by cocaine. However, unlike the other experiments on phenoxybenzamine antagonism, these were done with the antagonist added in the presence of a high concentration of agonist. The resulting antagonism was measured in terms of its rate of development

rather than the absolute level of inhibition achieved.

Many reports have indicated that agonists and reversible antagonists can prevent or reduce block of their own receptors by non-equilibrium antagonists (e.g. Nickerson & Gump, 1949; Nickerson, Henry & Nomaguchi, 1953; Furchgott, 1955; Innes, 1961). This indicates that the non-equilibrium antagonists first combine reversibly with the receptors. If such is the case, it might be asked why the same argument that applied to phentolamine in denervated tissues (see page 114) does not apply here: If agonist and antagonist are both in equilibrium, a change in affinity of the receptors for both should not be apparent when antagonism is measured.

The answer to this question lies in the parameter being measured. Whereas the final response determined under such conditions might not change, each component of the dynamic equilibrium will be governed by its own rate constant, with the result that the steady-state will be reached faster if the affinity of the receptors increases similarly for both drugs. If a level response to the agonist is obtained first, subsequently added antagonists will inhibit the response more rapidly, although the final level of response might be unchanged.

PART THREE

EXPERIMENTS BEARING ON EFFICACY

Our results strongly indicate that reserpine treatment increases the efficacy of the drug-receptor complex. This is based on the observation that there is a significantly greater maximum response to noradrenaline in spleen strips from reserpine-treated cats. None of our experiments indicate that denervation or cocaine cause any significant increase in the ability of the activated receptors to initiate contraction. It was hoped that experiments on calcium utilization would provide information regarding post-receptor events in supersensitivity. However, control values were extremely variable and the results were inconclusive. They provide only a hint that spleens from reserpine-treated cats might need less calcium in the bathing fluid to permit a 50 per cent contraction. Even assuming that this were true, more than one interpretation is possible. Entry of calcium into the cell might be enhanced, so that for a given outside concentration more would pass the membrane barrier. Alternatively, intracellular binding of the calcium might be decreased, so that for a given total intracellular concentration, less would be bound when the muscle is stimulated by agonists such as noradrenaline. Efficacy is generally regarded as a constant characteristic of receptors, with variability in the effectiveness of the drug-receptor complex being due to differences in efficacy between drugs. However, it is clear from our results that changes in post-receptor events may increase maximum responses.

Efficacy as a property of the receptors can only be examined simply if the maximum response requires full receptor occupancy, i.e.

if the stimulant is a partial agonist on the receptors then efficacy will be a function of the maximum response. If the receptor pool is larger than necessary to cause a maximum response, then a change in the receptors proper would not be seen as an increase in the maximum (see page 30). Under these conditions, the only way an increase in maximum response could be achieved would be by a quantitative increase in post-receptor processes which are activated by the receptors, or by an increase in available muscle fibres for contraction. This would amount to increasing the size of the effective receptor pool, since now a greater percentage of the total receptors would lead to a contraction of muscle elements.

These concepts are summarized schematically in Figure 26 and 27. Figure 26 shows how the maximum response could be increased when there are spare receptors. Under normal conditions a large number of individual receptors (R) form a receptor pool (R_t), only some of which (R_e) are effective when all are occupied by the agonist. The effective receptors are coupled by a series of events to a number of myofibrils (M) which represent an active fraction (M_a) of the total number of myofibrils (M_t). Stimulation of all the effective receptors leads to shortening of all the active myofibrils and causes a maximum response for the particular species of receptor. If spare myofibrils (M_s) exist, the provision of those links in the post-receptor chain of events which normally limit the maximum response would change some spare receptors (R_s) to effective ones and thereby increase the number of active myofibrils and the maximum response.

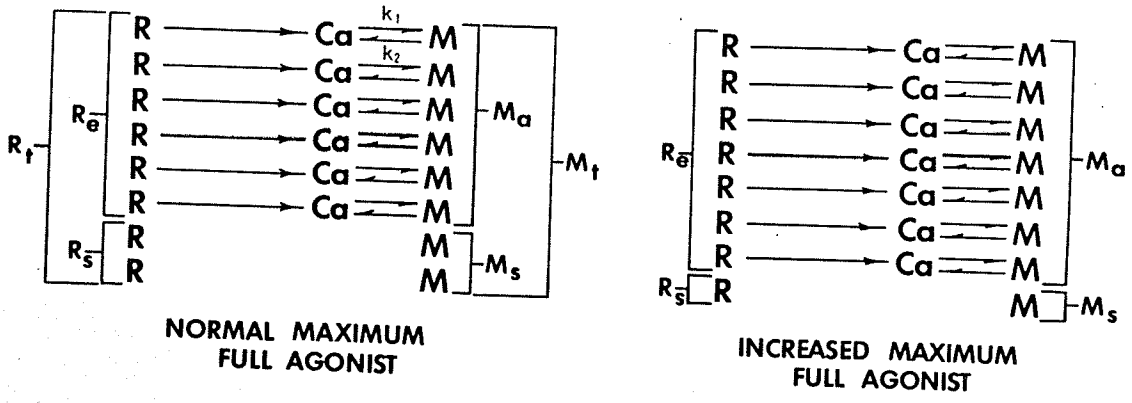

Fig. 26 SCHEMATIC MODELS TO ILLUSTRATE POSSIBLE POST-RECEPTOR CHANGES INCREASING THE MAXIMUM RESPONSE FOR A FULL AGONIST.

The following symbols are used: R_t- total receptor pool; R_e - effective receptors, or the number required to elicit a maximum response; R_s - spare receptors; M_t- total myofibrils; M_a- active myofibrils, or the number responding in a full contraction; M_s - spare myofibrils not activated in a full contraction. The arrows represent post-receptor events linking the activation of receptors to release of bound calcium, governed by rate constants for binding and release k_1 and k_2.

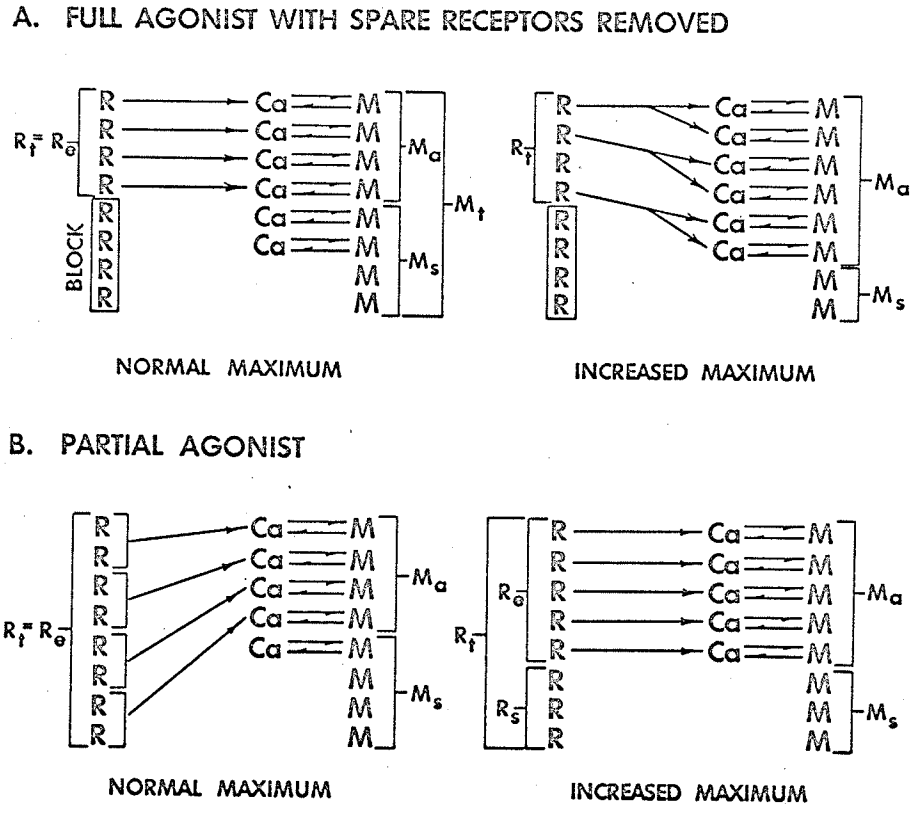

Fig. 27 SCHEMATIC MODEL TO ILLUSTRATE POSSIBLE RECEPTOR CHANGES INCREASING THE MAXIMUM RESPONSE.

See figure 26 for an explanation of symbols. B: A partial agonist whose normal maximum requires activation of the total receptor pool. A: A full agonist whose maximum response has been reduced by irreversible block of a fraction of the receptors, as indicated by the box.

In Figure 27, there are no spare receptors and $R_t = R_e$. This may be achieved artificially by irreversibly blocking all the spare receptors and then some, so that the maximum response is reduced (Fig. 27A). Alternatively, a partial agonist will normally provide such a weak stimulus per receptor that all the receptors are needed to cause a maximum response (Fig. 27B). In either case, M_a is less than M_t and there are spare myofibrils, whose activation requires only an adequate signal from stimulated receptors, as shown in Part B of the figure.

A. RESERPINE

We have found no other reports that reserpine increases maximum responses to noradrenaline. However, th experiments of Carrier & Holland (1965) indicate increased maximum responses after reserpine, although they fail to comment on this aspect of their results. They showed that isolated small branches of femoral artery from dogs treated with reserpine were supersensitive to injected noradrenaline. Figure 28, copied from their report, shows that reserpine decreased the threshold dose of noradrenaline required to cause a change in relative resistance in the isolated vessel. The most interesting change, however, is the increasing magnitude of the sensitization, indicated by the diverging curves. Unfortunately they did not complete their dose-response curves to the maximum response. However, they calculated the highest concentration added (10^{-4} g) to be about 3×10^{-6} g/ml, which would probably be in the upper region of the curve. If this is so, the divergence of the curves indicates that the maximum resistance change would

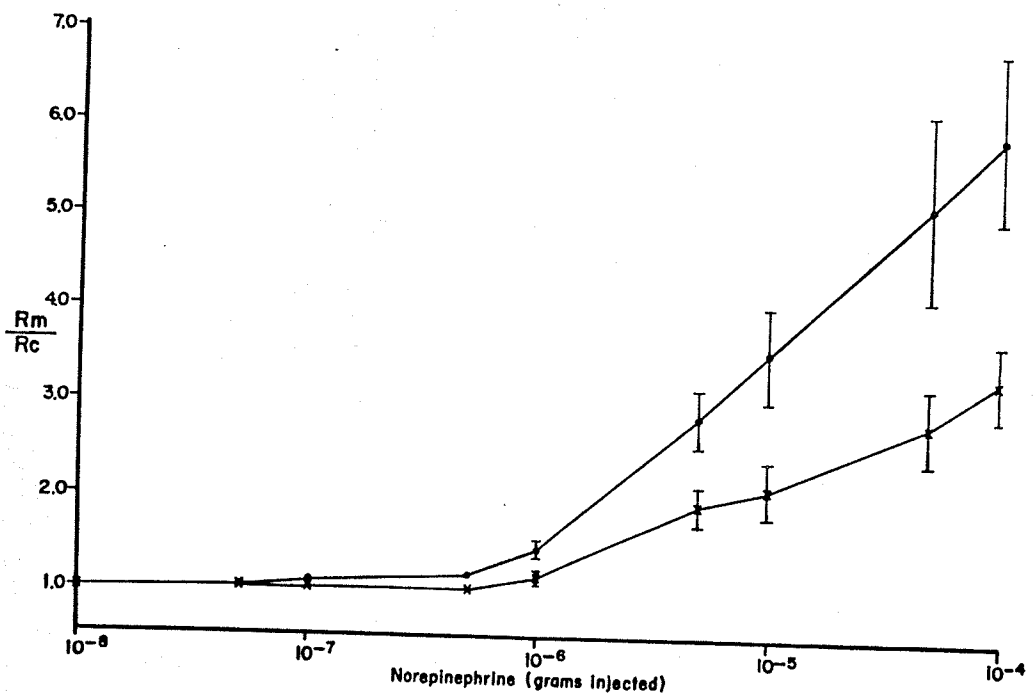

Fig. 28 APPARENT INCREASE IN MAXIMUM RESPONSE AFTER RESERPINE.

Copied from Holland & Carrier (1965). The figure shows resistance changes in isolated dog small artery segments. Ordinate: ratio of resistance after noradrenaline to resistance before noradrenaline. Abcissa: dose of noradrenaline injected. Each curve is the mean of 10 experiments, the upper one with vessels from reserpine treated dogs, the lower one with normal vessels.

have been greater in the vessel taken from a reserpine-treated animal. The authors did not state values for initial resistance in each group, so the increased change in resistance might be due to lower initial resistance after reserpine. However, the flow after 10^{-4} g of noradrenaline in the treated vessels was less than in the controls. Since hydrostatic pressure was constant, this would indicate that reserpine enables a greater absolute increase in resistance in response to this dose of noradrenaline.

Carrier & Holland also comment on a faster initial rate of onset, and a decreased rate of decay of the response in the treated vessels. They postulate as increased "effective" receptor area after reserpine, although in fact this represents only one of many possibilities. They note the theoretical possibility that loss of uptake of noradrenaline could account for the rate changes but discount this on histological grounds. However, their strongest evidence for effective receptor area increase lies in the maximum tissue response increase they show, although they fail to recognize it.

The effect of reserpine to increase maximum responses when there are spare receptors can only be explained by a post-receptor effect, such as shown in Figure 27. The provision of more links between receptors and myofibrils could increase the number of effective receptors (R_e). This could lead to an increase in the maximum contraction either by activating myofibrils which formerly failed to contract, or by promoting a general increase in the shortening of the myofibrils when activated. In either case, this would be schematically represented by a reduction in M_s

in Figure 27. Because our knowledge of the total post-receptor sequence is so vague, it is difficult to speculate on how reserpine might exert this effect. One possibility, mentioned above, is open to testing: the utilization of calcium for contraction may be altered so that it is either more readily available to the contractile elements, or there is more of it. For example, the calcium equilibrium between binding sites and actomyosin might be altered by a decrease in the firmness of attachment at the binding sites, or an increased affinity of the myofibrils for calcium so that the binding constant, k_2/k_1, (Figure 27) is decreased.

Alternatively, the absolute calcium concentration available for contraction might increase. In this connection Howell, Fairhurst & Jenden (1966) showed that calcium sequestered by preparations of fibres and granules made from rat diaphragm and gastrocnemius muscles increases after chronic denervation. Gutman & Sandow (1965) showed that caffeine is more effective in releasing calcium from the sarcoplasmic reticulum in denervated than normal muscle which suggests that the binding properties of the intracellular membranes are altered. In view of these findings is denervated skeletal muscle, it would be interesting to evaluate the possibility of similar changes in supersensitive smooth muscle, especially after reserpine.

Thus, while our experiments provide only a hint that intracellular calcium is used differently after reserpine, much more precise information could be obtained by studying isotopic calcium uptake and efflux. It is particularly important that experiments

be done to study changes in intracellular calcium concentration and binding in supersensitivity. Agents such as caffeine and the covalent ions nickel and cobalt are known to liberate calcium from binding sites (Frank, 1962). These could be used to test the firmness of calcium binding by measuring their effects on radiocalcium efflux in isolated preparations which have been previously depleted of their bound calcium, and then soaked in solutions containing radiocalcium. After similarly loading preparations with radiocalcium, another more physiological measure of binding would be the amount of radiocalcium liberated per stimulus by various chemical agents. Changes after treatment could be correlated with the development of supersensitivity and changes in maximum response. This would obviously have greater significance to the total post-receptor events than would the experiments with calcium-releasing agents.

B. COCAINE

Cocaine did not increase the full maximum response to noradrenaline. Nor did it alter the reduced maximum when the receptor pool was reduced by phenoxybenzamine. The strips in these experiments gave contractions to potassium which were greater than the noradrenaline maximum, indicating that phenoxybenzamine had not unspecifically depressed contractility. The results with noradrenaline indicated that cocaine does not alter the efficacy of the drug receptor complex. However, the experiments with isoproterenol gave some initially puzzling results. The maximum response of this partial agonist was greater after cocaine if the spleens were taken from

reserpine treated animals. The discrepancy between noradrenaline and isoproterenol was resolved when statistical analysis revealed that the change in maximum response to isoproterenol was significantly related to the initial response, and the sampling bias, which could not be appreciated at the time the experiments were done, had distributed those strips with lowest initial responses to the cocaine treated group when preliminary reserpine treatment was used, and to the control group when normal spleen strips were studied. There was therefore no significant effect of cocaine under either condition. These findings emphasize the danger in assuming that all random samples from a given population will be normally distributed within the population.

GENERAL DISCUSSION AND CONCLUSIONS

This investigation has provided an examination in one tissue of several processes in the stimulus-response sequence as they relate to the problem of supersensitivity. The widely accepted uptake hypothesis has been re-examined in an attempt to show a functional connection or separation between changes in uptake and the appearance of supersensitivity. Other processes which quantitatively influence the response to a stimulus have also been examined as they relate to supersensitivity, apparently for the first time.

We have shown that cocaine supersensitivity can be prevented by competition with other drugs, but that the ability of these drugs to protect against supersensitivity bears no relation to their known affinity for the uptake mechanism. By adding cocaine to a tissue after noradrenaline had been added and had equilibrated within the tissue to cause a steady-state response, we found that supersensitivity still occurred even though block of uptake could not increase the active concentration of noradrenaline at the receptors. Others have shown isoproterenol to have insignificant affinity for the uptake processes, yet when we added cocaine to spleen strips contracted by isoproterenol the potentiation was clear. These data, taken together, strongly argue against cocaine block of catecholamine uptake as a major factor in the supersensitivity it causes.

The result of the protection experiments suggested an action of cocaine on the alpha receptor in causing supersensitivity, and this possibility was strengthened when we found that both cocaine

and sympathetic denervation increased the degree of block of alpha receptors caused by phenoxybenzamine. Reserpine, however, did not share this effect, but did cause an increase in the maximum response to noradrenaline. Since there were spare receptors present, this must have been a post-receptor effect to increase the total number of effective receptors.

Our experiments with calcium restoration were designed to provide another approach to post-receptor events, but gave inconsistent results. There was a hint that reserpine could lower the amount of calcium needed to cause a half-maximum contraction; however, because of the variability, which was probably due to incomplete removal of bound calcium in the tissue, no weight can be attached to this observation. The possibility it raises must be investigated further, and we described experiments designed to more thoroughly evaluate calcium utilization in supersensitivity.

Our results with three different sensitizing procedures, cocaine, denervation, and reserpine, are internally consistent and not at variance with the observations of other investigators, although our interpretation is different. Thus abundant evidence from other investigators suggests that cocaine and chronic denervation cause a similar type of supersensitivity which is relatively specific for catecholamines and which causes the potency of noradrenaline to approach that of adrenaline. Our findings also suggest that cocaine and chronic denervation cause a similar type of supersensitivity; both cause changes which suggest an increase in receptor affinity, yet they have no effect on maximum responses.

Reserpine, on the other hand, is known to cause supersensitivity which differs qualitatively from that of cocaine and chronic denervation. Thus the potentiation is not specific for catecholamines and is less marked than that due to cocaine or denervation; in addition, the potency ratio for adrenaline and noradrenaline is unchanged. We found that reserpine did not affect alpha receptor affinity, which would be expected to cause a relatively specific supersensitivity, but that it did increase the maximum response which could be elicited from the tissue. Its effect in doing this was at the post-receptor level, and is therefore consistent with its observed lack of specificity in potentiating various agents.

In none of our experiments have we made direct measurement of catecholamine uptake in parallel with physiological recordings. This determination would be required to establish clearly that uptake changes did not influence our observations. We have therefore no direct proof that any process is functionally related to or separated from supersensitivity. However, our results do provide strong circumstantial evidence that catecholamine uptake and supersensitivity are not functionally related, and that we will have to look elsewhere for the answer to this problem. Since the uptake hypothesis is itself based on circumstantial evidence, our investigation should be evaluated on equal grounds.

Although the direct biochemical data are lacking there are several reasons which make it difficult to accept changes in uptake as an explanation for our results:

1. In the protection experiments, the one drug (phentolamine) which afforded complete protection against supersensitivity is known to have no appreciable affinity for the uptake process. In this respect, the direct measurement of uptake will be particularly important. If it can be shown that under the experimental conditions we have used, cocaine block of catecholamine uptake is not disturbed, this will conclusively demonstrate the separation between uptake and sensitivity.

2. Many processes combined determine the concentration of an active drug in final equilibrium with the receptors. However, it is difficult to conceive how this could be significantly affected by changes in uptake within an isolated tissue where the bathing fluid reservoir is much greater than the capacity of the uptake process. We showed that cocaine increased the noradrenaline response even after it had reached a steady-state. Several sites of action are possible for cocaine; however these data eliminate its known action to block catecholamine uptake sites as a plausible explanation for the supersensitivity it causes.

3. Isoproterenol effects on alpha receptors in the spleen are potentiated, yet this catecholamine has low affinity for the uptake processes in several tissues. In this connection, the uptake of isoproterenol by the spleen should be determined in experiments similar to those we have reported.

4. If an increased concentration at the receptors due to loss of uptake is the cause of supersensitivity, then when noradrenaline and phentolamine are present together in a tissue which is

deprived of uptake, the active noradrenaline concentration should be greater than control, but the phentolamine concentration should be unchanged because it is normally unaffected by uptake. This would result in an observed decrease in the phentolamine block of the alpha receptors. In our experiments with phentolamine, there was no change in the phentolamine block in denervated strips, suggesting that whatever change had occurred affected both agonist and antagonist similarly. This does not follow the pattern predicted by the uptake hypothesis.

5. Dose-ratio experiments which showed an increased antagonism by phenoxybenzamine after denervation may have been influenced by uptake of the antagonist. However, this is not likely in the maximum response decay experiments which showed an increased rate of inhibition by phenoxybenzamine after cocaine. Drugs which combine with the uptake mechanism do so competitively, and the high noradrenaline concentration in these experiments would be expected to keep phenoxybenzamine uptake low even in the tissues which received no cocaine. The changes observed after cocaine are therefore unlikely to be due to loss of uptake of phenoxybenzamine.

6. The effect of reserpine on maximum responses to noradrenaline is clearly incompatible with anything but a post-receptor effect since maximum response was increased when spare receptors were present and contraction height was limited by post-receptor events.

Considered in perspective, our observations on the isolated spleen lead to these conclusions:

1. Changes in catecholamine uptake are not functionally correlated to changes in sensitivity induced by cocaine.

2. Cocaine has an action on or near the alpha receptor, and both cocaine and sympathetic denervation increase the rate of block of alpha receptors by a non-equilibrium antagonist, which may represent an increased affinity of the receptors for drugs with which they combine.

3. Reserpine does not share the actions of cocaine and denervation on alpha receptors, but does increase the maximum response to noradrenaline, which can only be interpreted as an effect beyond the level of the receptors in the stimulus-response sequence.

In stating these conclusions, I recognize that the importance of uptake changes has not been finally resolved. Reasons have been stated in the discussion which I believe make it unlikely that uptake changes could have caused the results which led to the first two conclusions. Nonetheless, parallel measurements of catecholamine uptake must be made in future experiments before unqualified conclusions are permitted.

SUMMARY

The effects of denervation, cocaine and reserpine on cat spleen responses to catecholamines were studied in vitro. The experiments were designed to study possible changes in pre-receptor, receptor, and post-receptor events leading to contraction.

1. Noradrenaline and adrenaline responses were increased after cocaine. Noradrenaline, tyramine, phentolamine, isoproterenol, pronethalol, acetylcholine and histamine were tested to see if they prevented this effect. Phentolamine, which was very effective, is known to have low affinity for the catecholamine uptake sites in cat spleen. Other agents known to compete effectively with cocaine for the uptake process (noradrenaline and tyramine) had little or no effect. Drugs acting on adrenergic beta receptors (isoproterenol and pronethalol) and drugs acting on other receptor systems (acetylcholine and histamine) all had no effect.

2. After a submaximal steady-state contraction to noradrenaline was obtained, thus permitting the agonist to equilibrate with uptake mechanisms, cocaine clearly increased the response.

3. Submaximal steady-state contractions to isoproterenol, which has poor affinity for the uptake mechanisms, were also increased by cocaine.

These data indicate a functional separation between effects of cocaine on uptake and on tissue sensitivity. Allosteric transitions were suggested as a possible mechanism by which cocaine could cause potentiation.

4. Non-equilibrium block of alpha receptors by phenoxybenzamine was estimated from dose-ratios for noradrenaline. Reserpine treatment and denervation both caused supersensitivity to noradrenaline, but only denervation increased the dose-ratio. It was suggested that denervation and reserpine cause different types of supersensitivity, and that denervation might cause changes in receptor binding.

The rate of decay of maximum responses to noradrenaline caused by phenoxybenzamine was increased by cocaine, indicating a faster rate of receptor occupancy.

5. Competitive reversible block of alpha receptors by phentolamine was studied by measuring pA_{10} values for antagonism of noradrenaline. Neither denervation nor reserpine affected the antagonism, suggesting that a comparable change in receptor occupancy for both agonist and antagonist occurred in the supersensitive tissues.

6. Maximum responses were studied as an index of receptor efficacy. Reserpine caused the full maximum response to noradrenaline to increase. Because spare receptors were present, this was regarded as a change in post-receptor events to increase either the number of active myofibrils, or their contractility. Cocaine or denervation did not change the full maximum response to noradrenaline.

Cocaine did not increase the response to isoproterenol, a partial agonist where the response maximum is limited by the number of receptors, nor to noradrenaline after spare receptors were removed by phenoxybenzamine. These data indicate that cocaine supersensitivity involves no change in efficacy of the activated receptors.

Spleen strips depleted of calcium and exposed to noradrenaline were permitted to contract by adding graded concentrations of calcium to the bathing fluid. Sensitizing procedures caused no detectable change in the calcium concentration which allowed 50 per cent of the maximum contraction. The results were regarded as inconclusive because of failure to completely abolish the response to noradrenaline by calcium depletion.

The results indicate that specific supersensitivity to catecholamines bears no important functional relation to catecholamine uptake. Antagonist studies provide evidence that a change in receptor affinity may be involved. The data with reserpine indicate that it increases maximum responses by an effect on post-receptor events.

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