ACTIONS OF DEXAMPHETAMINE ON SMOOTH MUSCLE OF SPLEEN

A Thesis

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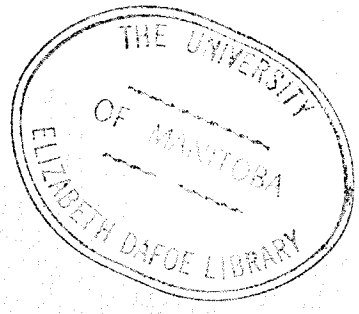

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by

Eugenie K. P. Tsang

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ABSTRACT

The purpose of this investigation was to find out how amphetamine acts on smooth muscle of spleen. An action on 5-hydroxytryptamine receptors and a direct and indirect action on adrenergic receptors have been described for amphetamine on various sympathetically innervated organs. Preliminary experiments showed unique response of the cat spleen strips to d-amphetamine, giving a small initial contraction while the drug was in the bath and a bigger long-lasting contraction after d-amphetamine was washed out. The results suggested four different actions of d-amphetamine: a major action due to release of noradrenaline, abolished by cocaine or prior treatment with reserpine; in high doses an antagonism of added or of released endogenous noradrenaline; supersensitization of the tissue to noradrenaline by low or subthreshold doses; a minor direct excitatory action which occurred only after large doses and which was not abolished by reserpine. The initial contraction is the resultant of these four actions, while the wash-out contraction is believed to be due to continued release of noradrenaline, the removal of the antagonistic effect of d-amphetamine, and the sensitizing effect of a small residual amount of d-amphetamine. The wash-out contraction did not occur in strips from spleen lacking noradrenaline due to treatment of the cat with reserpine or when release of noradrenaline was inhibited by cocaine. Reintroducing high or excitatory doses of d-amphetamine into the bath depressed the wash-out contraction.

Spleen strips from both normal and reserpine-treated cats were more sensitive to noradrenaline after wash-out of an excitatory dose of d-amphetamine. This is believed to be due to small amounts of d-amphetamine remaining in the tissue, since a subthreshold dose of d-amphetamine potentiated responses to noradrenaline in strips from normal and reserpine-treated cats and dogs and in denervated cat spleen.

The duration and other characteristics of supersensitivity caused by the subthreshold dose of d-amphetamine were also studied. The most striking findings were a gradual loss of effectiveness of repeated potentiating doses of d-amphetamine, sensitization to directly acting (e.g. adrenaline, nordefrine) and indirectly acting sympathomimetics (e.g. tyramine), and sensitization to drugs not acting on the adrenergic receptors (e.g. histamine, bethanechol, acetylcholine). Supersensitivity could not be related to monoamine oxidase inhibition, since nordefrine which is not destroyed by this enzyme was potentiated, and iproniazid, an inhibitor of monoamine oxidase, did not potentiate noradrenaline.

A group of sympathomimetic amines closely related to d-amphetamine in structure were tested for potentiation of noradrenaline and the structure-activity relationship was briefly explored. Hydroxyamphetamine, methamphetamine, phentermine, chlorphentermine, propylhexedrine and phenylethylamine caused initial and wash-out contractions characteristic of those described for d-amphetamine.

TO MY AUNT

CHEW-YING

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INTRODUCTION

A. General introduction and statement of the problem

Amphetamine, the dl-isomers of β-phenylisopropylamine, has been regarded as a sympathomimetic amine with strong C.N.S. activity ever since the 1930's. Its pressor effect on dog and man was first described by Piness, Miller and Alles (1930). Hartung & Munch (1931) attributed its prolonged action and oral effectiveness to the presence of a methyl group on the α-carbon of the β-phenylethylamine skeleton. In 1932, Alles reported a new method for synthesizing dl-β-phenylisopropylamine and in the following year (1933) he compared its action to that of adrenaline, finding it to be about 1/100 to 1/200 as effective but longer acting. At the same time he observed its bronchodilator, respiratory and C.N.S. stimulant effects. In 1934 (New and Non-official Remedies, 1934), the drug was introduced under the trade name of benzedrine for local application to constrict vessels of the nasal mucosae. A vast amount of literature concerning its cardiovascular, gastrointestinal, C.N.S. and anorectic effects, its clinical application and toxicity had appeared in the following two decades. Most of the papers were concerned with its C.N.S. effects, its clinical uses and limitations. The mechanisms of its peripheral actions, however, received relatively little attention.

Ten years ago, the peripheral effects of amphetamine were thought to be the result of a direct action of the drug on receptors of muscles and glands where adrenaline acts (Goodman & Gillman, 1955) yet certain differences in the action of amphetamine and adrenaline made this concept refutable, e.g. amphetamine exhibits tachyphylaxis (Alles, 1933; Winder, Anderson & Parke, 1948; Cowan, Cannon & Koppanyi,

1961; Hanna, 1960; Maengwyn-Davies, Cowan & Koppanyi, 1966) and, contrary to adrenaline and noradrenaline, amphetamine has less or no effect after administration of cocaine or sympathetic denervation (Drake, Renshaw, Modern & Thienes, 1939; Detrick, Millikan, Modern & Thienes, 1937; Tainter, 1929). Detrick and co-workers (1937) had questioned the true sympathomimetic nature of this drug since, according to him, "unquestioned sympathicotropic drugs, such as epinephrine are potentiated by cocaine, and their pressor action is reversed to depressor by ergotamine and similar ergot alkaloids. In our experiments, benzedrine action on blood pressure was decreased to approximately one-half both by ergotamine and by cocaine". This puzzle had passed unsolved through the years.

In 1958, Burn & Rand proposed a hypothesis for the mechanism of action of tyramine and sympathomimetics showing similar actions after reserpine treatment and sympathetic denervation. These other sympathomimetics include amphetamine, phenylethylamine and ephedrine. They called these noncatechol sympathomimetic amines "noradrenaline releasers", which presumably exerted their pressor and constrictor effects by releasing endogenous noradrenaline stores and the sympathomimetic effects of which were either diminished or abolished by denervation or reserpine treatment. Both denervation and reserpine treatment were known to deplete endogenous catecholamines (Goodall, 1951; Carlsson, Rosengren, Bertler & Nilsson, 1957; Burn & Rand, 1957; von Euler & Purkhold, 1951; Bertler, 1961 and many others). Vane (1960) proposed two mechanisms of action for amphetamine on rat stomach strips and rabbit duodenum.

He observed that rat stomach strip and rabbit duodenum bathed in Krebs solution was relaxed by adrenaline and noradrenaline but contracted to 5-hydroxytryptamine, phenylethylamine and dexamphetamine, the dextro isomer of amphetamine. He proposed that β-phenylethylamine and dexamphetamine caused contraction by action on tryptamine receptors (Gaddum, 1953) from evidence obtained by methods on specific antagonism and specific desensitization (Cheema, 1966). Vane showed that 1) actions of both 5-hydroxytryptamine and dexamphetamine responses were reduced to the same extent in the presence of bromo-lysergic acid diethylamide (10^{-7} g/ml), a specific antagonist for 5-hydroxytryptamine; 2) phenoxybenzamine also reduced the contractions produced by amphetamine and 5-hydroxytryptamine to the same extent; 3) prolonged exposure of the tissue to tryptamine or 5-hydroxytryptamine desensitized the tissue to the action of not only 5-hydroxytryptamine but also dexamphetamine and 4) prolonged exposure of the tissue to dexamphetamine desensitized the tissue to the action of 5-hydroxytryptamine or tryptamine. In contrast, he observed that rat stomach strip and rabbit duodenum superfused with blood from a donor cat was profoundly relaxed by amphetamine and this relaxation was very long lasting. Vane explained this effect of dexamphetamine on the blood-bathed tissues as due to local release of noradrenaline in the tissue itself since blood-bathed stomach strips prepared from rats pretreated with reserpine reacted in a similar way as the untreated strips bathed in Krebs solution, i.e. by contraction instead of relaxation. He also suggested that

when the tissue was bathed in Krebs solution, the stores of noradrenaline were either depleted or in some way made inaccessible to the releasing amines so that the excitatory effect of β-phenylethylamine and dexamphetamine on tryptamine receptors was unmasked. He further showed that replenishing the stores with noradrenaline caused the tissue to relax to tryptamine and dexamphetamine.

Innes (1963), using isolated preparations of rat stomach, dog retractor penis, rabbit aorta, rabbit uterus and guinea-pig ileum, confirmed Vane's conclusion that dexamphetamine acted on the same receptor as 5-hydroxytryptamine, and at the same time showed that adrenaline and 5-hydroxytryptamine acted on different receptors by using the technique of specific receptor protection and cross-protection. He showed that receptor protection with adrenaline against block by phenoxybenzamine did not extend to dexamphetamine and 5-hydroxytryptamine, whereas 5-hydroxytryptamine and dexamphetamine gave cross-protection against phenoxybenzamine in a dose which usually caused block. He also showed that dexamphetamine did not act through release of endogenous catecholamines in dog retractor penis, rabbit aorta and rabbit uterus, which were contracted by both adrenaline and dexamphetamine because responses to dexamphetamine were not reduced after cocaine or in preparations from animals pretreated with reserpine.

In a previous paper, Innes (1962) had reported that 5-hydroxytryptamine contracted cat spleen strips by acting on adrenaline receptors, again on evidence provided by techniques on specific antagonism, specific receptor protection, cross-protection and

specific desensitization (for techniques see Cheema, 1966). How then does amphetamine act in the spleen strips? Since the spleen is an organ with high noradrenaline content it seems quite possible that amphetamine acts by release of noradrenaline.

Preliminary experiments done in this laboratory showed that d-amphetamine caused contraction of cat isolated spleen strips bathed in Krebs-Henseleit solution. Long-lasting contraction was seen when d-amphetamine was removed from the bath by changing bath fluid. This was unusual since most of the isolated tissue preparations (e.g. rabbit aorta, guinea-pig ileum, rat stomach strip, dog urinary bladder, etc.) would relax when the agonist to which they had contracted had been washed out of the bath. Moreover, the spleen strips always relaxed after removal of acetylcholine, histamine, 5-hydroxytryptamine, adrenaline and noradrenaline, all of which caused contraction of the tissue, therefore contraction of the tissue after removal of the drug seems to be peculiar to amphetamine and the spleen strips. We have therefore investigated more closely the action of d-amphetamine on this particular preparation, the spleen strips. The effects of a number of the congeners of d-amphetamine have also been tested.

As we shall be concerned with storage, release and uptake of noradrenaline, action of a partial agonist and potentiation or supersensitivity, it is thought appropriate to give a brief review on each of these topics.

B. Stores of catecholamines and indirectly acting sympathomimetic amines.

1) Early association of catecholamines with sympathetic nerves:

In 1904, Elliot, on finding the striking resemblance of the action of adrenaline and stimulation of sympathetic nerves, suggested that adrenaline might be "the chemical stimulant liberated on each occasion when the impulse arrives at the periphery" (quoted from Dale, 1960). Later, Löewi (1921) gave evidence that adrenaline was liberated from the frog heart upon stimulation of its sympathetic nerves. Cannon & Rosenblueth (1933) showed that, in mammals, mixtures of adrenaline and noradrenaline were liberated from sympathetic nerves. Bülbring & Burn (1949) also found mixtures of noradrenaline and adrenaline liberated during splanchnic nerve stimulation of spinal cat. von Euler (1946) reported noradrenaline was the substance present in spleen extract. Peart (1949) and Mann & West (1951) confirmed this by analysing the blood collected from the spleen, liver, uterus and intestine of cats after sympathetic nerve stimulation and found noradrenaline to be the major neurohumor.

Lissack (1939) extracted sympathetic substances in nerve trunks and this extractable "sympathin" in nerve trunks disappeared upon nerve degeneration (Cannon & Lissack, 1939). Later, Goodall (1951) confirmed their observation by describing the disappearance of extractable noradrenaline after degeneration of post-

ganglionic sympathetic nerves in the heart, while von Euler & Purkhold (1951) described it for the spleen, liver, the kidney and salivary glands of the sheep. These workers also showed that upon functional regeneration of the nerves, noradrenaline content of the tissues rose to their predenervated level. More recently, Brown & Gillespie (1957) measured noradrenaline in the venous effluent of the blood perfused cat's spleen after electrical stimulation of the splenic nerve.

2) Indirect evidence for the existence of catecholamine stores.

a) Actions of reserpine and tyramine:

Although tyramine and adrenaline both exert similar effects, it was known 50 years ago that under the influence of cocaine the action of adrenaline was potentiated whereas that of tyramine was abolished (Tainter & Chang, 1927). Other differences of action between these amines were observed in denervated tissues, e.g. Burn & Tainter (1931) reported that tyramine and ephedrine had no or little action on the denervated cat iris although it was super-sensitive to adrenaline; Burn (1932) showed that removal of the stellate ganglion which innervated the cat's foreleg caused tyramine to lose it vasoconstrictor action but not adrenaline; Bülbring & Burn (1938) reported that tyramine had no effect on denervated nictitating membrane of cat.

The difference in the action of adrenaline and tyramine was not explained until the late 1950s when reserpine was discovered to deplete endogenous catecholamine in tissues. Bertler, Carlsson &

Rosengren (1956), Burn & Rand (1957) observed that injection of reserpine into the blood stream of experimental animals caused the extractable noradrenaline from the heart and thoracic aorta to decrease profoundly. It was soon found that reserpine caused disappearance of noradrenaline content in various tissues, e.g. the spleen and iris of the cat (Burn & Rand, 1959); dog aorta (Burn & Rand, 1958); ear skin of rabbit (Burn & Rand, 1958a).

Carlsson and co-workers (1957) showed that the pressor effect of tyramine was lost in a cat treated with reserpine. The disappearance of noradrenaline content in tissues that were denervated or from reserpine-treated cats and the inactivity of tyramine under both circumstances seemed to fit in well together. 1958, Burn & Rand showed that after reserpine treatment, tyramine and some noncatecholamines (such as phenylethylamine, ephedrine and amphetamine) no longer contracted the nictitating membrane and the spleen of spinal cat, and did not exert their pressor effect. Reinfusion of noradrenaline into the blood stream of these animals restored the actions of tyramine which was given after the effects of noradrenaline had passed off. Burn & Rand therefore advanced the hypothesis that tyramine and similar noncatecholamines normally act by liberating noradrenaline stored in the tissue.

Since then, a great deal of evidence has supported the hypothesis of Burn and Rand and fortified the concept of the existence of "stores" of noradrenaline in the sympathetic nerves. Only a few examples will be given. Intravenous injection of tyramine in cats has been shown to increase the plasma concen-

tration of adrenaline and noradrenaline in the lower aorta (Lockett & Eakins, 1960). Other experiments showing the increase of plasma or perfusate levels of noradrenaline after the administration of tyramine have been described by Burn & Burn (1961); Lindmar & Muscholl (1961); Stjärne (1961); Axelrod, Gordon, Hertting, Kopin & Potter (1962); Chidsey, Harrison & Braunwald (1962) and many others. Hertting, Axelrod & Patrick (1961) reported depletion of stores of noradrenaline in various tissues after the administration of tyramine while Potter, Axelrod & Kopin (1962) described depletion of stores from the rat heart by a series of tyramine-like agents. Readers are referred to Muscholl (1966) for a review on indirectly acting sympathomimetic amines.

Much evidence shows cocaine affects tyramine as denervation does (Fleckenstein & Bass, 1953; Fleckenstein & Stockle, 1955; MacMillan, 1959) and it is now believed that cocaine has the effect of blocking release of noradrenaline from the stores so that it prevents or antagonizes the pharmacological actions of tyramine (Tainter & Chang, 1927; MacMillan, 1959; Lindmar & Muscholl, 1961; Hertting, 1965). This blockade has been reported to be dose related and can be overcome by increasing the dose of tyramine (Hertting & Hess, 1962).

b) Uptake and release of labelled amines:

i) Retention of labelled amines in whole animals.

Studies on the uptake and release of labelled amines con-

firmed the ability of sympathetic innervated structures to take up and store these amines. Experiments carried out by injecting H^3-adrenaline and H^3-noradrenaline into the mouse and then estimating the disappearance of the labelled amines from the whole mouse showed a fast and slow phase of disappearance. Rapid disappearance occurred in the first 5 minutes, associated mainly with O-methylation of H^3-noradrenaline. Part of the H^3-noradrenaline (30-50% of injected dose) remained in the tissue for several hours or days and was slowly metabolized (Whitby, Axelrod & Weil-Malherbe, 1961; Potter & Axelrod, 1963). This second slow phase of disappearance indicated that part of the labelled amines were bound to the tissue in some form or other and being protected from rapid metabolism (Axelrod & Tomchick, 1960). Tyramine, phenylethylamine, d-amphetamine and ephedrine markedly increased the rate of disappearance of these labelled amines, thus suggesting release or interference with the binding mechanism (Axelrod & Tomchick, 1960).

ii) Uptake and accumulation of labelled amines in isolated tissues or organ.

The ability of various tissue slices to take up and concentrate H^3-noradrenaline from the incubation medium have been reported by many workers (e.g. Brodie, Dengler, Titus & Wilson, 1960; Dengler, Spiegel & Titus, 1961; Wilson, Murray & Titus, 1962). Since these tissue slices could accumulate the labelled catecholamines against the concentration gradient, specialized transport mechanisms have been postulated to be involved. Kopin,

Hertting & Gordon (1962), Iversen (1963) and Callingham & Burgen (1966) reported uptake and concentration of labelled noradrenaline by the isolated rat heart.

iii) Uptake of labelled amines in denervated tissues.

The above experiments did not give evidence whether the uptake is mainly associated with sympathetic nerves or other tissue binding sites. Hertting, Axelrod, Kopin & Whitby (1961) found that denervated tissues had less ability to take up and bind labelled noradrenaline to a great extent. Hertting (1965) showed that the amount of endogenous and labelled noradrenaline taken up and bound in denervated cat heart (10 days after removal of both stellate ganglia) was much less (about 1/5 to 1/10 of normal) than in the innervated heart, indicating a great part of the uptake and storage was associated with nervous tissue. However, a small portion of extraneuronal binding of H^3-noradrenaline was shown to exist in chronically denervated tissues (Fischer, Kopin & Axelrod, 1965).

iv) Release of bound exogenous amines upon nerve stimulation.

The bound exogenous H^3-noradrenaline in the cat spleen, was shown by Hertting & Axelrod (1961) to be released upon stimulation of the splenic nerve, just as the endogenous adrenergic transmitter. Other labelled agents such as α-methyldopa and α-methylnoradrenaline were taken up in amounts stoichiometrically comparable to the amount of noradrenaline displaced (Porter, Totaro & Burcin, 1966) and liberated from the heart during nerve stim-

ulation (Muscholl & Maitre, 1963). Labelled metaraminol and guanethidine have also been shown to displace noradrenaline from their stores and were released as a false neurohumor upon nerve stimulation (Crout & Shore, 1964; Boullin, Costa & Brodie, 1966).

v) Drugs altering uptake and binding of labelled amines.

Drugs which change the concentration of endogenous catecholamines also alter the uptake and binding of administered radioactive noradrenaline. Of the extensive work on this subject only a few examples will be mentioned. In most experiments of this type, drugs were given before or after H^3-noradrenaline was given to the animal or isolated tissues. H^3-noradrenaline content in the tissue or whole animal was estimated at a constant time interval after the administration of H^3-noradrenaline. Cocaine and imipramine reduced the amount of H^3-noradrenaline uptake in the heart, spleen and adrenalines only if given before H^3-noradrenaline indicating blockade of uptake (Whitby, Hertting & Axelrod, 1960; Muscholl, 1961 and many others). Reserpine, tyramine, amphetamine and guanethidine lowered the H^3-noradrenaline concentration in the tissues whether they were given before or after H^3-noradrenaline (Axelrod, Hertting & Potter, 1962; Hertting, Axelrod & Patrick, 1962 and others); thus showing these drugs may release as well as interfere with the uptake of noradrenaline (for a review see Muscholl, 1965). A great number of sympathomimetics including d-amphetamine and other agents have been shown to interfere with the uptake of H^3-noradrenaline in

the rat isolated heart (Iversen, 1964; Burgen & Iversen, 1965).

3) Direct evidence for the existence of noradrenaline stores in sympathetic nerves.

Although the labelled-amine technique provided direct evidence for nervous tissue to take up and release noradrenaline, yet this is no real evidence for the existence of discrete stores of noradrenaline within the nerve or nerve endings. Direct evidence for their existence has come from histochemical studies and isolation of subcellular nerve granules from sympathetic nerves.

a) Histochemical studies:

With the help of autoradiography and electromicroscopy, Wolfe, Potter, Richardson & Axelrod (1962) provided actual visualization of H^3-noradrenaline being concentrated within granulated vesicles at the autonomic nerve terminals. This was also described by Marks and co-workers (1962). Reserpine has been shown with this technique to significantly reduce the uptake and storage of radioactive amines in the heart and vas deferens of mice (Samarajski, Marks & Webster, 1964).

Intraneuronally located noradrenaline has also been shown by fluorescent method devised by Falck & Torp (1962). This is based on the condensation of primary catecholamines with formaldehyde and their rapid transformation in the presence of protein to fluorescent dihydroquinoline derivatives. The entire adrenergic neurone has been shown to fluoresce with high intensity of

fluorescence at the nerve terminals (Falck, 1962; Owman & Falck, 1965; Malmfors, 1965).

b) Subcellular nerve granules:

Blaschko & Welch (1953) first demonstrated that catecholamines are stored within chromaffin granules in the adrenal medulla. Some years later, von Euler & Hillarp (1956) and von Euler (1958) isolated subcellular granules containing noradrenaline from the pressed juice of bovine splenic nerves. This corresponds well with the histochemical findings. It has been suggested that catecholamines in these nerve granules are bound to adenosine triphosphate as do the amines in the chromaffin granules from the adrenal medulla (Schümann, 1958). Goodall & Kirshner (1958) showed that these granules synthesize noradrenaline from dopamine. These granules release noradrenaline spontaneously but slowly at 37°C. Tyramine in a dose 3 µg/ml increased the amount of noradrenaline released from these granules (von Euler & Lishajko, 1960a; von Euler & Lishajko, 1961). The effects of a number of drugs on the uptake and release of noradrenaline in this system have also been described by von Euler & Lishajko (1965). Since the granular fraction of noradrenaline represents only 30 per cent of the total amount of noradrenaline in the nerve homogenates the existence of bound and free pools of noradrenaline has been suggested (von Euler & Lishajko, 1961). Readers are referred to Stjärne (1966) for a review of this type of work and a comparison of the properties of adrenal medullary and nerve granules.

C. Action of a partial agonist.

Knowledge about receptors and drug-receptor interaction is still speculative and limited. However, many of the early conclusions and assumptions have been re-evaluated and modified (Nickerson, 1965). It is now known that tissue activation is not directly proportional to the fraction of total receptors occupied. Nickerson (1956) and Stephenson (1956) demonstrated the existence of "spare receptors" and that maximum responses to many potent agonists require only a very small fraction of the total number of receptors. Different drugs when combined with the same receptors may have varying capacities to initiate a response; thus when two drugs are producing equal responses they may be occupying different proportions of the receptors (Ariens, 1954; Stephenson, 1956), therefore the activity of a drug is not simply a measure of its affinity (Reuse, 1949) for the receptors but also its intrinsic activity (Ariens, 1954). The affinity reflects the readiness of the drug to combine or interact with the receptors, thus depending on the concentration, dissociation constant, Van der Waal's and other interacting forces. The intrinsic activity measures the ability of the drug-receptor complex to initiate a response. Stephenson (1956) used the term "efficacy" for "intrinsic activity" which was described by Ariens and co-workers.

An agonist with high intrinsic activity may produce a maximum response even though it occupies a small fraction of the receptors, whereas an agonist with low intrinsic activity may produce only a fraction of the maximum response although it is

occupying all the receptors. If the two agonists act on the same set of receptors, the one with low intrinsic activity can antagonize the effect of that with a higher intrinsic activity by competing for the receptors. Compounds with low intrinsic activity can thus act as agonist and antagonist and were termed "partial agonists" by Stephenson (1956). The maximum response to a partial agonist with all receptors occupied is less than that of a full agonist. Ariens & co-workers described them as showing "dualism in action" (Ariens, 1954; Ariens, van Rossum & Simonis, 1957). Stepwise change in the chemical structure of an agonist has been shown to result in a gradual change from full agonist to partial agonists and then to antagonists (van Rossum & Ariens, 1959a; van Rossum & Ariens, 1959b; van Rossum, 1962; Ariens & Simonis, 1960; Ariens, 1960, 1963).

A partial agonist may sometimes act as a synergist to a pure agonist, depending on the concentrations: at low concentrations of both or of the pure agonist, it acts as a synergist but at higher concentrations of the agonist it acts as a competitive antagonist (van Rossum, 1960).

D. Supersensitivity.

A number of procedures and drugs is known to cause supersensitivity of excitable tissues (striated and smooth muscles, nervous tissues such as autonomic ganglia, spinal neurones, etc.) resulting in increased responses to drugs or other stimuli (see review by Cannon & Rosenblueth, 1949). This discussion will be confined

to supersensitivity to adrenergic stimuli in structures of sympathetic innervation.

Within the past half century, there have been numerous reports on drugs and procedures which enhance responses to sympathomimetic amines and attempts have been made to explain the underlying mechanisms although none has been fully successful. The terms "potentiation", "synergism", and "supersensitivity" have been used in different senses by various authors and there has been some confusion as to the proper use of these terms (for review see Veldstra, 1956). "Supersensitivity" is now generally used to describe increase responses of an excitable tissue to stimuli under various circumstances, with no indication of what the underlying mechanism may be. Similarly the terms "potentiation" (of a drug or response) and "enhancement of response" will be used here with no attempt to imply a knowledge of the mechanism involved.

A full account of the historical background and the various aspects of studies in supersensitivity would be inordinately long, and therefore this section will be limited to a brief review of the most important procedures and drugs that produce supersensitivity, a few salient points of historic interest, and some current concepts in connection with the cause of supersensitivity.

The study of supersensitivity dates back to the 19th century, when Budge (1855) discovered that cat iris responded to asphyxia with "paradoxical dilatation" after its sympathetic nerves had degenerated and later when Lewandowsky (1899) reported that

chronically denervated cat nictitating membrane responded to adrenal extract with increased magnitude. Thus, supersensitivity was first associated with surgical denervation (for review see Cannon & Rosenblueth, 1949). Two types of denervation supersensitivity have later been distinguished 1) supersensitivity developed after chronic postganglionic denervation (usually termed "denervation" or "chronic denervation"), i.e. division of the postganglionic nerves to an effector organ several days before its sensitivity is tested; 2) supersensitivity developed after chronic preganglionic denervation (usually termed "decentratization" or "chronic decentralization"), i.e. division of the preganglionic nerve supply to the effector days before its sensitivity is tested (Cannon, 1939, Cannon & Rosenblueth, 1949).

Frölich & Löewi (1910) reported that cocaine increased mydriatic and pressor responses to adrenaline. Since then many drugs have been reported to increase smooth muscle responses to adrenergic stimuli. For example, ephedrine potentiated adrenaline in blood vessels of rabbit ear (Gaddum & Kwiatkowski, 1938); several other sympathomimetic amines increased the cardiovascular response to adrenaline (Jang, 1940); low doses of adrenergic blocking agents such as ergotoxin, yohimbine and piperoxan increased responses to adrenaline in the rabbit ear (Jang, 1941); chronic administration of phenoxybenzamine caused supersensitivity of cat nictitating membrane (Nickerson & House, 1958). Reserpine has been reported to cause supersensitivity to catecholamines in cat nictitating membrane, perfused vascular beds and isolated

artery strips 24 hours after administration (Burn & Rand, 1958, 1958a). Xylocholine, bretylium, and guanethidine, agents which block transmitter release from the adrenergic neurones also caused supersensitivity (Exley, 1957; Boura & Green, 1959; Maxwell, Plummer, Schneider, Povalski & Daniel, 1960; Abbs, 1962). Recently, imipramine and desmethylimipramine have been reported to have potentiated the action of noradrenaline on various sympathetically innervated organs (Soffer & Gyermek, 1961; Sigg, Soffer & Gyermek, 1963; Schaeppi, 1960).

Various hypotheses have been put forward to explain the phenomenon of supersensitivity or the underlying mechanisms. Cannon (1939), noting the unspecificity of denervation supersensitivity suggested that supersensitivity was due to changes in permeability of the denervated tissues to stimulating ions or substances. Burn & Robinson (1953) reported that denervation supersensitivity could be correlated with the fall in monoamine oxidase (MAO) concentration in muscle and suggested that supersensitivity to adrenergic stimuli was a result of MAO inhibition, but this hypothesis could not explain later findings that inhibition of MAO by agents such as iproniazid did not potentiate noradrenaline-induced contractions of smooth muscle (Griesemer, Barsky, Bragstedt, Wells & Zeller, 1953; Furchgott, 1955; Kamijo, Koelle & Wagner, 1956). More recently, catechol-O-methyl transferase (COMT) was found to be an important enzyme in the normal destruction of catecholamines (Axelrod, 1957). Although some COMT inhibitors (e.g. pyrogallol) increased catecholamine responses

which were not increased by potent MAO inhibitors (Bacq, Gosselin, Dresse & Renson, 1959) yet inhibition of COMT could not be the cause of supersensitivity after cocaine or denervation, since cocaine or denervation did not inhibit COMT (Wylie, Archer & Arnold, 1960) and O-methylation of catecholamines was actually increased after denervation (Potter, Cooper, William & Wolfe, 1965).

In 1953, Fleckenstein & Bass, remarking that both denervation and cocaine reduced the continuous discharge of noradrenaline from postganglionic nerve fibers, postulated that increased sensitivity of effector cells was due to decreased "accommodation" of the effector cells to noradrenaline. Burn & Rand (1959) gave reasons to abandon the "enzyme hypothesis" and put forward a hypothesis related to that of Fleckenstein & Bass. Since either reserpine or chronic denervation resulted in loss of tissue stores of noradrenaline, they suggested that continuous slow release of noradrenaline from the intact stores normally keeps the sensitivity of the effector tissue low, and thus the removal of this inhibitory source would result in supersensitivity. This hypothesis has later been challenged and criticized since the increase in sensitivity to exogenous noradrenaline in the muscles of the nictitating membrane could not be related to the decrease in stores of noradrenaline in the membrane (Kirpekar, Cervoni & Furchgott, 1962; Trendelenburg & Weiner, 1962; Fleming & Trendelenburg, 1961).

Deformation of receptors by combination of sensitizing agents

with allosteric sites, thereby increasing the affinity for catecholamines, has also been suggested as a cause of supersensitivity (Maxwell, Plummer, Daniel, Schneider & Povalski, 1958; Maxwell, Plummer, Povalski, Schneider & Coombs, 1959; Maxwell, 1965).

The most recent and currently popular explanation is the "uptake hypothesis" which is based on the assumption that uptake into the nerve endings and intraneuronal storage sites normally diverts a great part of noradrenaline away from its site of action and that supersensitivity to adrenergic stimuli is due to blockade or impairment of tissue catecholamine uptake, thus leaving a larger amount of agonist to reach the receptor site (Trendelenburg & Weiner, 1962; Kirpekar et al, 1962; Trendelenburg, 1963, 1966). However, there are many instances where the relationship between impairment of uptake and supersensitivity cannot be established. For example, prevention of noradrenaline uptake cannot explain the unspecific supersensitivity of the denervated nictitating membrane to acetylcholine (Trendelenburg, 1963) and to barium (Schmidt & Fleming, 1964). Many drugs resembling cocaine in preventing uptake of noradrenaline fail to increase response to noradrenaline (Trendelenburg, 1966) while other drugs (such as metanephrine, normetanephrine) potentiate noradrenaline in various tissues but do not appear to inhibit labelled noradrenaline (Furchgott, 1966). So far no single hypothesis can fully explain the complexity of supersensitivity.

E. The spleen strips.

The experimental object used in this work is the isolated spleen strip. Although the spleen strip has been little used as an *in vitro* preparation until recently, Sherrington first described it in 1919, in a manual of practical exercises for physiology students as a preparation of "exsected spleen" and showed a tracing of its contraction to adrenal extract. Federicq (1929) observed contraction of dog spleen strip to adrenaline, and Vairel (1933) reported isolated splenic capsules of dog, rabbit, tench and frog contracted to adrenaline. Saad (1935) extended similar observations to man, cat, guinea-pig, rat and buffalo. He further showed that the contractions were abolished by ergotamine. More recently, Bickerton, Rockhold & Micalizzi (1962) used isolated cat spleen strips to assay adrenergic blocking drugs, and Innes (1962) showed that 5-hydroxytryptamine and adrenaline acted on the same receptors in cat spleen strips while histamine and acetylcholine each acted on its own specific receptors. Bickerton (1963) reported catecholamines produced a contraction of the cat spleen strips through common receptors (the α-adrenergic receptors) and their order of potency was: adrenaline > noradrenaline > isoproterenol. Most recently, Bickerton, O'bleness & Rockhold (1966) made use of contractions of cat spleen strips to catecholamines to explain discrepancy between theoretical and experimental dose-response curves. Kizaki and Abiko (1966) reported that pronethalol, a beta-blocker, inhibited the contractions to adrenaline, acetylcholine and isoproterenol of spleen strips from cats and rabbits,

and that this was not due to the specific blocking action of pronethalol on β-receptors.

The rich sympathetic innervation of the spleen (von Euler, 1956) and smooth muscles which readily respond to sympathomimetic amines make the spleen strip a good in vitro system for the study of sympathetic mechanisms. Tissue stores of noradrenaline are abundant and can be released or depleted by nerve stimulation or by drugs such as reserpine and tyramine (Peart, 1949; von Euler, 1956; Brown & Gillespie, 1957; Burn & Rand, 1959; von Euler & Lishako, 1960; Stjärne, 1961).

The spleen strips are generally quiescent when suspended in the organ baths before and between tests. Only on rare occasions is a strip met which exhibits slow intrinsic movements (slow rhythmic contractions and relaxations; each contraction and relaxation lasting about 10-20 minutes, with amplitudes of about 3-5 mm). One drawback about the spleen strips is that the smooth muscles are interspersed among rich reticuloendothelial tissues whose effects on drug distribution and muscle contraction are not known.

METHODS

Preparations of Spleen Strips

Cat Spleen Strips

Cats of either sex, weighing 1.5 to 2.5 kg, were killed by a blow on the head. Spleens were quickly removed and immersed in cold (4^{o} C) Krebs-Henseleit solution (Table I). Strips 2.5 cm long and 2-3 mm wide were cut from the edge of the spleen. Each strip was suspended in an individual organ bath containing 10 ml of Krebs-Henseleit solution kept at 37^{o} C and bubbled with 95% O_2 and 5% CO_2. Isotonic responses against 1 g tension at six times magnification were recorded on a kymograph.

Dog Spleen Strips

Spleen strips were prepared in the same way from dogs of either sex (4-6 kg).

All strips were allowed to equilibrate for one hour before drugs were tested. Bathing fluid was changed at 10-15 minute intervals during this time. During the course of the experiment drugs which caused contraction were generally washed out of the bath as soon as maximum responses were attained, usually within 3-10 minutes.

TABLE 1

Composition of Krebs-Henseleit solution	Concentration	
	g/l	mM
NaCl	6.9	118.0
KCl	0.35	4.7
$CaCl_2$	0.28	2.5
KH_2PO_4	0.16	1.1
$MgSO_4$	0.14	1.2
$NaHCO_3$	2.20	25.0
Glucose	2.00	11.0

Treatment with Reserpine

Cats or dogs whose noradrenaline stores were to be depleted were given reserpine (1 mg/kg) intraperitoneally 24 hours before the experiment.

Chronic Denervation of Cat Spleen

Cats weighing 2.8 to 3.4 kg were anaesthetized with sodium pentobarbital (35 mg/kg) intraperitoneally. The spleen was exposed by a midline incision in the abdomen, with precautions to maintain asepsis. Connective and nervous tissues surrounding the branches of the splenic arteries were carefully stripped off. The abdominal wound was repaired and the cats were allowed to recover for 14 days. The spleen strips were then prepared in the usual manner.

Drugs

Sympathomimetic Amines

Table 2 lists the sympathomimetic amines and the source of their supply. Stock solutions (1 mg/ml of base) were made in 0.01 M HCl. On the morning of use, the stock solutions were diluted as required with acidified 0.9% NaCl solution. Phenethylamine, being a liquid, was diluted first to 1 mg/ml with 0.01 M HCl for stability, then to the required concentration with acidified 0.9% NaCl solution.

Other Agonists

Other agonists used were acetylcholine chloride (Calbiochem.),

histamine diphosphate (Nutritional Biochemical Corporation), and 5-hydroxytryptamine creatinine sulphate (Calbiochem). Stock solutions and final dilutions of these drugs were made up as were the sympathomimetic amines.

Other Drugs

Stock solutions of cocaine hydrochloride (British Drug Houses) (1 mg/ml of the salt) and of iproniazid phosphate (Hoffman La Roche) (1 mg/ml of the salt) were made in distilled water. Suitable dilutions were made daily in 0.9% NaCl solution.

A solution of reserpine (Ciba) for intraperitoneal injection was prepared by dissolving 100 mg reserpine in a mixture of 2 ml glacial acetic acid, 2.5 ml propylene glycol, 2.5 ml ethanol and distilled water to 20 ml volume. The solution contained 5 mg of base/ml. Prenylamine (Segontin gluconate, Hoechst) was supplied as a 5% solution of the base. Suitable dilutions were made daily in 0.01 M HCl before use.

All stock solutions were stored at 4^{o} C. Drug concentrations mentioned in the text refer to the final concentrations in the bath fluid in terms of the free base. Concentrations of cocaine and iproniazid were expressed in terms of the salts.

TABLE 2

l-Adrenaline bitartrate	(Sterling-Winthrop)
d-Amphetamine sulphate	(Smith Kline & French)
l-Amphetamine sulphate	(Smith Kline & French)
Chlorophentermine hydrochloride	(Warner-Chilcott)
Diethylpropion hydrochloride	(Merrell Company)
Ephedrine sulphate	(Nutritional Biochemicals Corp.)
Hydroxyamphetamine hydrobromide	(Smith Kline & French)
Mephentermine sulphate	(John Wyeth and brother)
Metaraminol bitartrate	(Merck Sharp & Dohme)
Methamphetamine hydrochloride	(Burroughs Wellcome)
Methoxamine hydrochloride	(Burroughs Wellcome)
Methoxyphenamine hydrochloride	(Upjohn Company)
l-Noradrenaline bitartrate	(Calbiochem)
Nordefrin (Cobefrine hydrochloride)	(Sterling-Winthrop)
Norsynephrine hydrochloride	(Sterling-Winthrop)
Phendimetrazine Bitartrate	(Delmar Chemical Limited)
Phenethylamine hydrochloride	(Sterling-Winthrop)
Phentermine hydrochloride	(Strasenburgh)
Phenylpropanolamine hydrochloride	(Merck Sharp & Dohme)
Phenylpropylmethylamine hydrochloride	(Merrell Company)
Propylhexedrine hydrochloride	(Smith Kline & French)
Tyramine hydrochloride	(Calbiochem)

RESULTS

A. Response of Spleen Strips to d-Amphetamine

1) Characteristics of response of cat and dog spleen strips to d-amphetamine:

a) Initial contraction.

d-Amphetamine, 10^{-5} to 10^{-4} g/ml, caused contractions of 5-35 mm in 25 cat spleen strips and 10 dog spleen strips (1-4 strips from each of 15 cats and 2-4 strips from each of 3 dogs). The contractions took 5-15 minutes to reach their maximum. The threshold dose varied from 3×10^{-7} to 3×10^{-6} g/ml, giving 1-2 mm contraction.

b) Wash-out contraction.

In the above experiments, d-amphetamine was washed out of the bath as soon as the contraction reached its maximum and the bath fluid was then changed every 10-15 minutes. The tissue, instead of gradually relaxing, contracted further (Fig. 1a). The contraction reached a height of 10-40 mm greater than the original contraction in 15-30 minutes, then remained constant for 1-2 hours before the tissue began to relax. This contraction occurred whether washing was done by draining and refilling the bath or by overflow method. The tissue took another 30-40 minutes to relax fully. This long-lasting wash-out contraction occurred in all strips tested with an effective dose of d-amphetamine, and in 4 cat spleen strips (2 each from 2 cats) it lasted up to 5 hours.

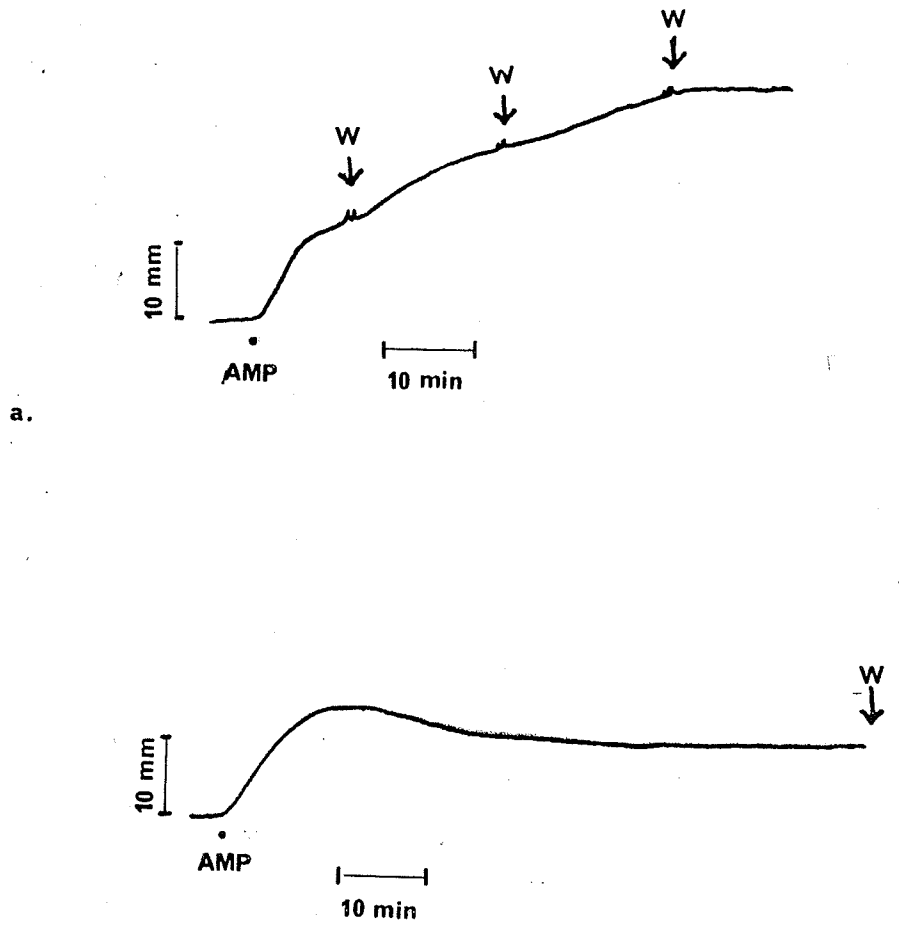


Fig. 1 Response of cat spleen to d-amphetamine.

a. d-Amphetamine 5 x 10^{-5} g/ml added at AMP and washed out 10 min later. Washes at W.

b. d-Amphetamine 5 x 10^{-5} g/ml added at AMP, left in the bath for 70 min. Wash at W.

Experiments were later done to see whether contractions to the same amounts of d-amphetamine (10^{-5} to 10^{-4} g/ml) would reach the height of the wash-out contraction if the drug were kept in the bath for a longer time. Six cat spleen strips (2 each from 3 cats) and one dog spleen strip were used. When d-amphetamine was left in the bath for 30-70 minutes, the contraction, after reaching a maximum, tended to decrease a little (2-3 mm) then became constant in 15-30 minutes. Removal of d-amphetamine (after 30-70 minutes) from the bath did not cause any further contraction (Fig. 1b). l-Amphetamine behaves in a similar manner to its d-isomer.

Characteristic initial and wash-out contractions to d-amphetamine (10^{-4} g/ml) were also observed in two rat spleen strips.

c) Relationship between onset of wash-out contraction and time of exposure to d-amphetamine.

A direct relationship between the time of exposure to d-amphetamine and the time taken for the onset of wash-out contraction was observed in four experiments. In two experiments, 4 spleen strips from the same cat were set up at the same time. d-Amphetamine (5×10^{-5} g/ml) was left in the baths for (1) until maximum contraction was obtained, (2) 5-10 minutes after maximum was reached, (3) 15 minutes after maximum was reached and (4) 20-25 minutes after maximum was reached. In each of the other two experiments, only two strips were tested, treated as (1) and (4).

Upon washing, (1) showed immediate wash-out contraction; in

(2) the onset of wash-out contraction was delayed 5-10 minutes; in (3) and (4) there was a long delay of 1-2 hours before the wash-out contraction. Wash-out contractions in (1) and (2) lasted 2-3 hours, and in (3) and (4) only 30-60 minutes. The sizes of the wash-out contractions in (3) and (4) are much smaller than those in (1) and (2). There seems to be an inverse relationship between exposure time and the size of the wash-out contraction (Table 3). The sizes of wash-out contraction of (1) and (2) are significantly greater than those of (3) and (4); $0.05 > p > 0.01$ by Student's t test. Since the initial contractions were not of the same height, the percentage increase of the wash-out over the initial contractions were taken for comparison.

It was later found (in 8 strips from 2 cats) that no wash-out contraction occurred when the exposure to d-amphetamine continued for 30-40 minutes after maximum contraction was reached (see p. 47).

2) Effect of reserpine on response to d-amphetamine.

Burn & Rand (1958) found that amphetamine caused little rise of blood pressure and no contraction of the nictitating membrane in reserpine treated cats. Its effect on the nictitating membrane was restored by infusion of noradrenaline. They suggested that amphetamine, like tyramine, acts by release of endogenous noradrenaline stores. It seems quite possible here that the wash-out contraction is due to release of noradrenaline. Experiments were therefore done to see if wash-out contractions occurred in strips obtained from animals treated with reserpine (1 mg/kg 24 hr before the experiment) to eliminate noradrenaline stores.

TABLE 3

LENGTH OF EXPOSURE TO d-AMPHETAMINE AND SIZE OF WASH-OUT CONTRACTIONS

Experiment No.	(1) Wash at Attaining Maximum			(2) Wash 5-10 mins. After Maximum			(3) Wash 15 mins. After Maximum			(4) Wash 20-25 mins. After Maximum		
	Contraction (mm)		% Increase	Contraction (mm)		% Increase	Contraction (mm)		% Increase	Contraction (mm)		% Increase
	Initial	Wash-Out		Initial	Wash-Out		Initial	Wash-Out		Initial	Wash-Out	
1	4	16	300	4	6	50	3	4	33	3	0	0
2	8	15	88	10	30	200	8	10	25	5	8	60
3	10	47	370							5	12	114
4	3.5	13.5	288							4	6	50

The percentage increase of the wash-out over the initial contractions in (1) + (2) compared by Student's t test, are significantly greater than in (3) + ($\overline{4}$), $0.05 > p > 0.01$.

Each experiment was on two strips, one from a reserpine treated cat and one control strip from a normal cat. Six such pairs of strips were used (2 strips each from 3 reserpine treated cats and 3 untreated cats). A test dose of tyramine (10^{-5} g/ml) was first given to all strips. Strips which did not respond to this dose of tyramine were assumed to be depleted of stored noradrenaline, since tyramine acts by release of noradrenaline (Carlsson et al., 1957; Burn & Rand, 1958, and many others). None of the strips from reserpine treated animals responded to 10^{-5} g/ml tyramine; the control strips from normal animals gave a contraction of 11-25 mm to a small dose of tyramine (10^{-6} g/ml) (Fig. 2).

The strips were then exposed to noradrenaline (10^{-7} g/ml) to test their viability. This induced a contraction of 6-35 mm in all strips. The strips from reserpine-treated animals in fact showed a greater sensitivity to noradrenaline. The mean height of contraction to noradrenaline (10^{-7} g/ml) with standard error for the 6 strips from reserpine treated animals was 21.6 ± 2.3 mm; that for the 6 control strips was 14.3 ± 5 mm. Therefore the ineffectiveness of tyramine on strips from reserpine treated cats was not due to general depression of tissue responses by reserpine. Experiments done in the same laboratory indicated that this dose of noradrenaline (10^{-7} g/ml) does not replenish the stores enough for even large doses of tyramine to cause contraction (Karr, 1966).

d-Amphetamine (10^{-5} - 3×10^{-4} g/ml) was then introduced and left in the bath for 5-15 minutes. The control strips showed a

5-15 mm initial contaction which reached its peak within 5-15 minutes, and a wash-out contraction (4-36 mm greater than initial) after removal of d-amphetamine. Four of the strips from cats treated with reserpine did not contract although d-amphetamine was left in the bath for 5-15 minutes. The other two strips gave a contraction of 1-3 mm. No wash-out contraction occurred when d-amphetamine was removed (Fig. 2).

Similarly d-amphetamine (10^{-5} - 10^{-4} g/ml) caused no initial or wash-out contraction in 5 strips from two dogs treated with reserpine (1 mg/kg 24 hr before experiment).

On two occasions, d-amphetamine (10^{-3} g/ml) caused a contraction of 2-3 mm but no wash-out contraction in strips from reserpine-treated cats after a smaller dose (3×10^{-4} g/ml) had been ineffective.

Two experiments on receptor protection were then done, according to the method described by Innes (1962), to see if d-amphetamine would protect the adrenaline receptors from blockade by phenoxybenzamine in strips from reserpine-treated cats. d-Amphetamine (10^{-4} g/ml) prevented phenoxybenzamine (5×10^{-8} g/ml) from blocking noradrenaline (10^{-7} g/ml) but not histamine (10^{-6} g/ml).

The above results suggest that d-amphetamine has at least two actions on the spleen strips; 1) a direct action which is not abolished by reserpine and 2) an indirect action through release of noradrenaline which is abolished by treatment with reserpine. The absence of wash-out contraction in all strips lacking noradren-

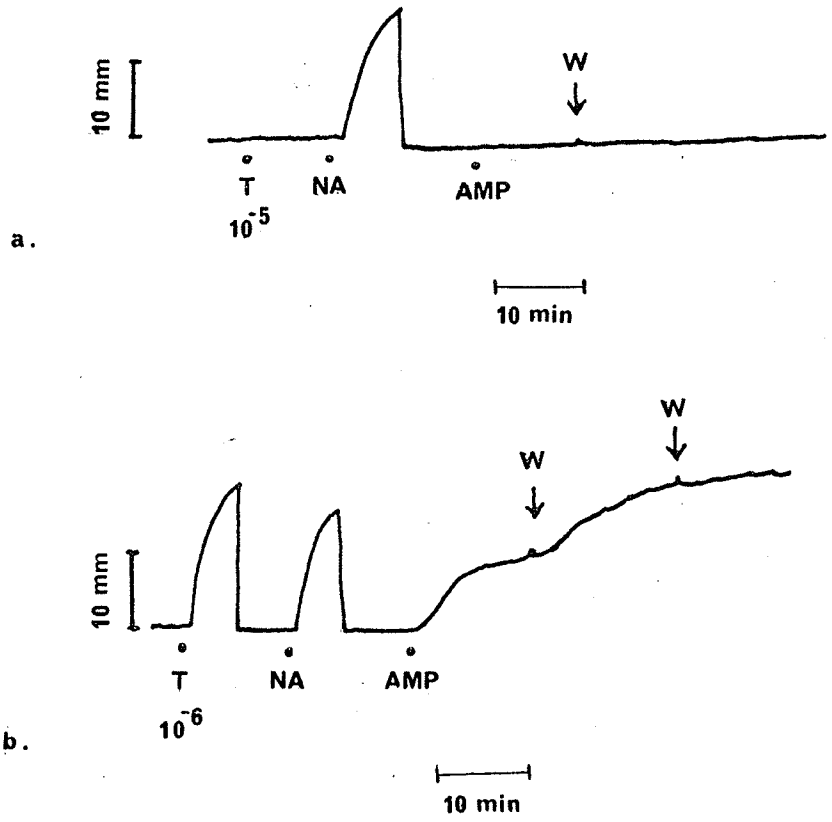


Fig. 2 Responses to tyramine, noradrenaline and amphetamine in strips from reserpine-treated and control cats.

a. Strip from reserpine-treated cat contracted neither to tyramine (T, 10^{-5} g/ml) nor to d-amphetamine (AMP, 10^{-4} g/ml) but gave a contraction of 18 mm to noradrenaline (NA, 10^{-7} g/ml). d-Amphetamine wash-out after 10 min did not give wash-out contraction. Wash at W.

b. Strip from control cat contracted to tyramine (T, 10^{-6} g/ml), noradrenaline (NA, 10^{-7} g/ml) and d-amphetamine (AMP, 10^{-4} g/ml). Washing out d-amphetamine (at W) caused further contraction of the strip. In this and in all subsequent experiments as soon as contractions to agonists (except d-amphetamine) reached maximum the drum was stopped till strips had returned to their original length.

aline stores suggests strongly that wash-out contraction is due to release of endogenous noradrenaline.

3) Antagonism of noradrenaline by d-amphetamine.

If wash-out contraction was due to noradrenaline release, d-amphetamine should be antagonistic to noradrenaline since wash-out contraction appeared only after removal of d-amphetamine, therefore d-amphetamine reintroduced into the bath after wash-out should reduce or depress the wash-out contraction. In 4 cat spleen strips (2 each from 2 cats) the initial contractions to d-amphetamine (10^{-5} g/ml) were 5-12 mm and the wash-out contractions were 5-11 mm greater than the initial contraction. d-Amphetamine reintroduced into the bath (10^{-5} g/ml) decreased the contractions to their initial heights (5-12 mm). In two strips, d-amphetamine was then quickly removed and wash-out contraction was again seen. In the other two strips d-amphetamine was not washed out but higher doses (10^{-4} - 10^{-3} g/ml) of d-amphetamine were added until the strips returned to their uncontracted length, presumably completely desensitized to d-amphetamine.

Six experiments were then done to see if d-amphetamine would antagonize added as well as endogenous noradrenaline. Each experiment consisted of one control and one strip from reserpine-treated cats. Six spleen strips from 3 normal cats and 6 from 3 cats treated with reserpine were used. At least two approximately equal responses to noradrenaline (10^{-7} g/ml) were first recorded before the strips were exposed to d-amphetamine (10^{-5} or 5×10^{-5} g/ml).

When the responses to d-amphetamine in the control strips had reached a maximum, noradrenaline (10^{-7} g/ml) was added. In these experiments, d-amphetamine was not washed out of the bath until the strips had fully responded to noradrenaline. The contractions to noradrenaline before d-amphetamine exposure were 10-25 mm but those in the presence of d-amphetamine (10^{-5} or 5×10^{-5} g/ml) were only 1-2 mm, reduced to about 1/10 in size. This antagonism was observed in both the normal strips and strips from reserpine-treated animals (Fig. 3).

The results of these experiments and the fact that very high doses of d-amphetamine (10^{-4} - 10^{-3} g/ml) caused only a small contraction (1-3 mm) in strips from reserpine-treated animals indicated that d-amphetamine is at most a partial agonist having a much lower intrinsic activity than that of noradrenaline.

The control dose of noradrenaline used in these and subsequent experiments was either 10^{-7} or 3×10^{-7} g/ml. These doses caused contractions of about 10-30 mm in cat and dog spleen strips and were known, from experiments on dose-response curves of noradrenaline, to lie on the straight part of the curves. Fig. 4 shows the mean partial dose-response curve of noradrenaline for 8 spleen strips, 2 each from 4 cats. Fig. 5 shows the partial dose-response curve of noradrenaline obtained from a dog spleen strip.

4) Effect of d-amphetamine wash-out on responses to other agonists.

The contractions to various agonists were tested during the

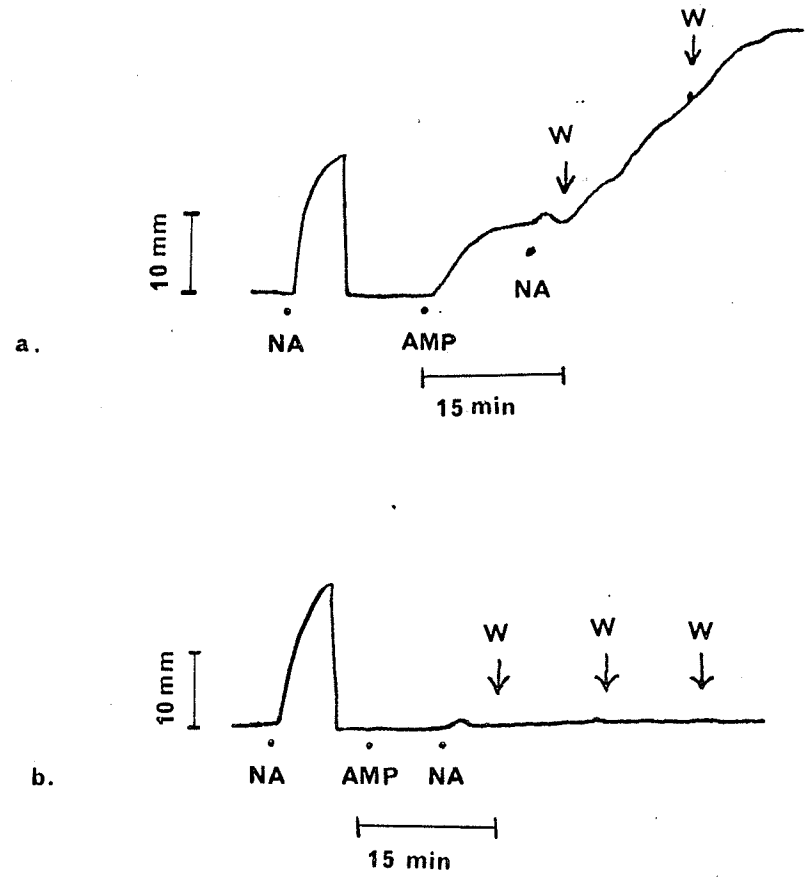


Fig. 3 Antagonistic effect of d-amphetamine on noradrenaline response in cat spleen strips.

a. Strip from control cat gave a contraction of 17 mm to noradrenaline (NA, 10^{-7} g/ml). d-Amphetamine (AMP, 5 x 10^{-5} g/ml) was left in the bath for 15 min and a contraction of 8 mm occurred. Noradrenaline (NA, 10^{-7} g/ml) tested while amphetamine was in bath gave only 1.5 mm contraction. ⟵⟶ = d-amphetamine kept in bath. Wash at W. There was a large wash-out contraction.

b. Strip from a reserpine-treated cat gave a contraction of 16 mm to noradrenaline (NA, 10^{-7} g/ml). d-Amphetamine (AMP, 5 x 10^{-5} g/ml) was left in the bath for 15 min. Noradrenaline (NA, 10^{-7} g/ml) tested while amphetamine was in bath gave only 1 mm contraction. Wash at W. There was no wash-out contraction.

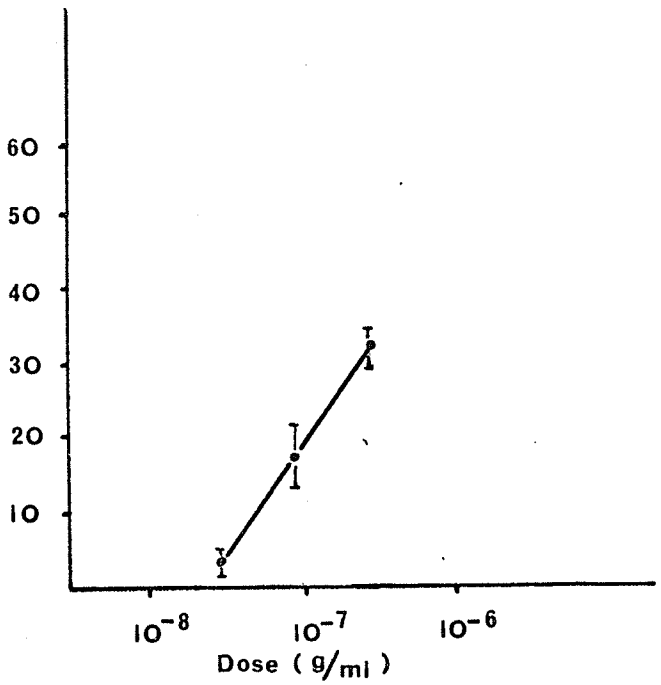


Fig. 4 Mean partial dose-response curve of noradrenaline for 8 spleen strips of 4 cats (with standard errors).

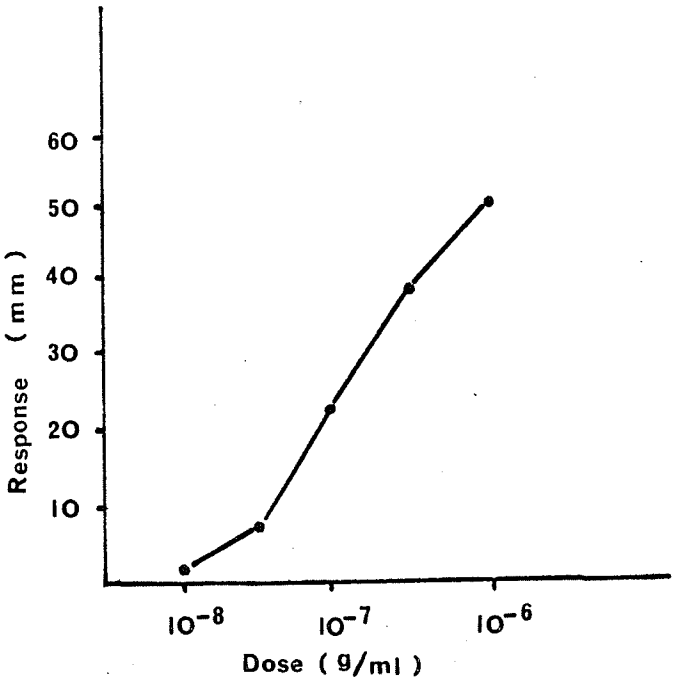


Fig. 5 A partial dose-response curve of noradrenaline obtained from a dog spleen strip.

prolonged wash-out contraction due to d-amphetamine. Eight spleen strips, 2 each from 4 cats, were used. Contractions to histamine (10^{-6} g/ml), acetylcholine (3×10^{-6} g/ml), noradrenaline (10^{-7} g/ml) and 5-hydroxytryptamine (10^{-5} g/ml) were recorded before the strips were exposed to 10^{-5} g/ml d-amphetamine. Responses to these agonists were tested again when the wash-out contraction had reached a plateau. Histamine, acetylcholine and noradrenaline caused contractions comparable to the control ones (1-2 mm difference, which is within the limits of biological variation) whereas 5-hydroxytryptamine relaxed the tissue (Fig. 6).

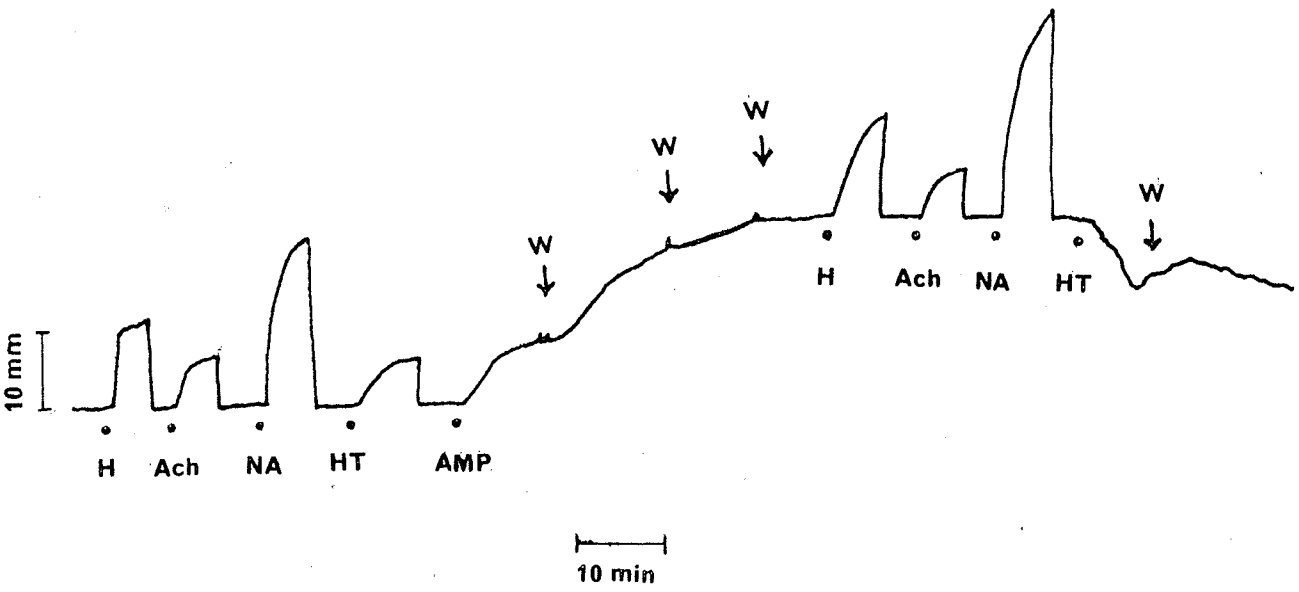


Fig. 6 Effect of d-amphetamine wash-out on responses to histamine (H), acetylcholine (Ach), noradrenaline (NA) and 5-hydroxytryptamine (HT).

Control responses to histamine (H, 10^{-6} g/ml), acetylcholine (Ach, 3 x 10^{-6} g/ml), noradrenaline (NA, 10^{-7} g/ml) and 5-hydroxytryptamine (HT, 10^{-5} g/ml) were first recorded at intervals of 15-35 min, with bath fluid changed after each test. d-Amphetamine (5 x 10^{-5} g/ml) was then added and removed at W after 6 mins. 30 mins later, individual response to H (10^{-6} g/ml), Ach (3 x 10^{-6} g/ml), NA (10^{-7} g/ml) and HT (10^{-5} g/ml) were again recorded. All agonists except 5-hydroxytryptamine gave contractions of heights comparable to those of the control responses.

B. Potentiation of Noradrenaline After d-Amphetamine Wash-Out

1) Strips from reserpine-treated cats and dogs.

While repeating tests on the antagonism of noradrenaline by d-amphetamine in spleen strips from reserpine-treated cats (p. 37) we found that responses to noradrenaline were greatly increased after the antagonistic dose of d-amphetamine was washed out. Thus d-amphetamine, after being washed out, left the strips more sensitive to noradrenaline than in the control period.

Four strips from 3 cats treated with reserpine were used to confirm the above observation. Noradrenaline contraction (10^{-7} g/ml) tested before the strips were exposed for 5-10 minutes to d-amphetamine (5×10^{-5} or 10^{-4} g/ml) were 9-20 mm (mean = 15.5 ± 2.5 mm S.E.); those tested 15-30 minutes after d-amphetamine wash-out were 28-42 mm (mean = 34.3 ± 3.5 mm S.E.), an increase of 87-200% (mean = 129 ± 35.7 S.E.). In two strips a second dose of noradrenaline (10^{-7} g/ml) was tested 60 minutes after wash-out; potentiation was still present, but somewhat reduced (Fig. 7).

Responses to noradrenaline (10^{-7} g/ml) were increased by 38% and 50% in two spleen strips from reserpine-treated dogs 20 minutes after wash-out of d-amphetamine (3×10^{-4} g/ml).

2) Strips from normal cats.

The observation that the contraction of strips from normal cats to noradrenaline was the same during a wash-out contraction as during the earlier control period (p. 41) did not necessarily

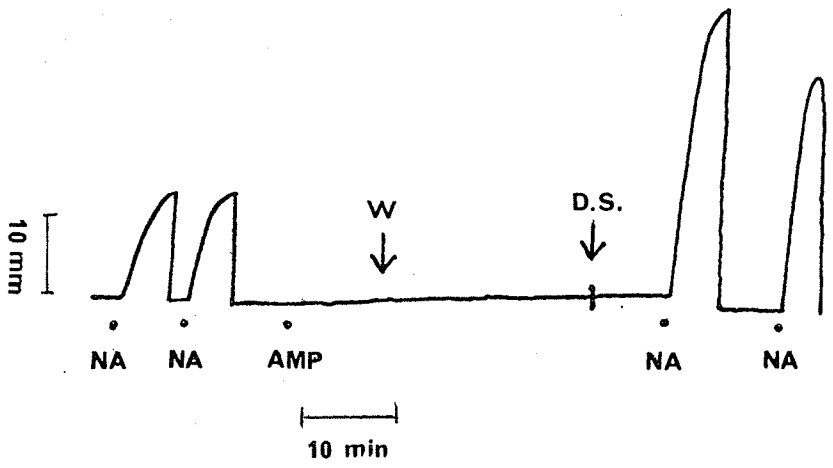


Fig. 7 Enhancement of noradrenaline response after d-amphetamine wash-out (a spleen strip from a reserpine-treated cat).

NA, responses of the spleen strip to noradrenaline (10^{-7} g/ml). d-Amphetamine (AMP, 10^{-4} g/ml) was left in the bath till W (10 min). Noradrenaline (NA, 10^{-7} g/ml) was tested 30 min and 60 min after W. Drum was stopped at D.S.

indicate that the sensitivity of the spleen to noradrenaline was unchanged during the wash-out contraction. At that time the total contraction appears to be due entirely to noradrenaline, partly endogenous released by amphetamine and partly exogenous added to the bath. Therefore the response to the exogenous noradrenaline will depend on the amount of endogenous noradrenaline released in relation to the dose-response curve of the tissue to noradrenaline; hence similarity between responses before and during wash-out contraction is probably fortuitous. The sensitivity to noradrenaline during wash-out contraction therefore could not be simply tested. Accordingly noradrenaline was tested as soon as the tissue had fully relaxed from the wash-out contraction, usually 2-3 hr. after wash-out. Two types of experiments were done, one where d-amphetamine was washed out as soon as the contraction reached maximum, the second with the wash-out 30-40 minutes after maximum contraction was reached. Noradrenaline was tested before addition of d-amphetamine and immediately the wash-out contraction had completely disappeared (first type).

a) Wash at maximum contraction.

Eight strips, 2 each from 4 cats, took 2-3 hr. to relax fully after wash-out of d-amphetamine (10^{-5} - 5 x 10^{-5} g/ml). At this point responses to noradrenaline (10^{-7} - 3 x 10^{-7} g/ml) were greater than during the control period in all eight strips (Fig. 8). Contractions during the control period varied from 9-22 minutes, falling in the lower quarter of the dose response curve. Con-

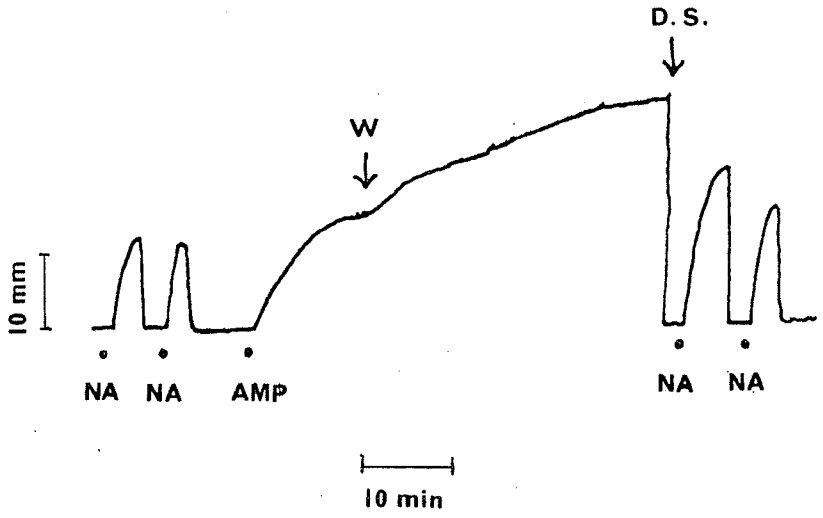


Fig. 8 Enhancement of noradrenaline response after d-amphetamine wash-out (normal tissue).

Fig. shows two control responses of a normal cat spleen strip to noradrenaline (NA, 10^{-7} g/ml). d-Amphetamine (AMP, 10^{-4} g/ml) left in the bath for 12 mins gave an initial contraction and a wash-out contraction greater than the initial. Wash is indicated at W. The strip took 3 hours (bath fluid changed every 10-15 mins) to relax fully. Noradrenaline response (NA, 10^{-7} g/ml) tested as soon as tissue relaxed to base line was greater than the control (82% increase). A second noradrenaline response (NA, 10^{-7} g/ml) tested 40 mins later was potentiated less than the previous one (36% increase).

D.S. = drum stopped. Time from W to next NA test was 3 hours.

tractions after the end of the wash-out contraction were 17-33 mm; the mean increase was 71%. An additional strip was used as a time control; noradrenaline was tested at the same times as in the strips exposed to d-amphetamine, but no d-amphetamine was added. There was no change in the responses to noradrenaline tested 3 hours apart.

A second noradrenaline response was tested $3\frac{1}{2}$ hours after d-amphetamine wash-out in 3 strips. Potentiation still occurred but was less marked. Fig. 8 shows the result of a typical experiment.

Two strips of dog spleen gave similar results. Control responses to noradrenaline (10^{-7} g/ml) were 8 and 13 mm; responses after d-amphetamine (10^{-4} g/ml) wash-out were 13 and 27 mm. The percentage increases were 62% and 108%.

b) Wash at 30-40 minutes after maximum contraction.

When d-amphetamine (10^{-5} - 5×10^{-5} g/ml) was left in the bath until 30-40 minutes after maximal contraction was reached, no wash-out contraction occurred in 8 strips, 2 each from 4 cats. During the first hour after wash-out the relaxation was very slow but the strips were fully relaxed after 2-3 hours. Since there was no wash-out contraction, noradrenaline was tested shortly after wash-out of d-amphetamine in 3 experiments. Each experiment was done on 3 spleen strips from the same cat. Control responses to noradrenaline (10^{-7} g/ml) were recorded. d-Amphetamine (5×10^{-5} g/ml) was then added and kept in the bath for 30-40 minutes after

maximum was attained. Noradrenaline (10^{-7} g/ml) was then tested again only once in each strip. In the 3 strips the tests were at 5, 10 and 20 minutes after removal of d-amphetamine. All strips gave responses to noradrenaline with 100-200% increase over the control responses (Table 4). Fig. 9 shows a typical record. Potentiation also occurred when the strip had fully relaxed. This was observed in 3 strips.

TABLE 4

INCREASED CONTRACTION TO NORADRENALINE AFTER WASH-OUT OF d-AMPHETAMINE (d-AMPHETAMINE REMAINED IN BATH 30-40 MINS AFTER MAXIMUM INITIAL CONTRACTION WAS REACHED).

Percentage Increase Over Control Response

Experiment	Strip No.		
	1	2	3
	Time After Wash-Out		
	5 min	10 min	20 min
1	140	100	144
2	210	200	260
3	150	152	160

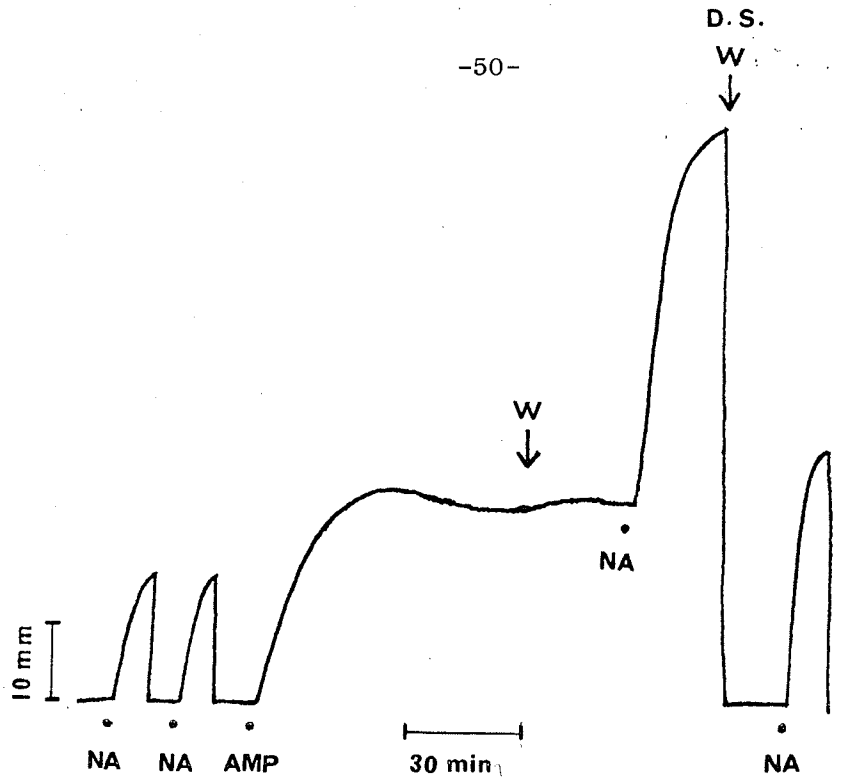


Fig. 9 Enhancement of noradrenaline response in normal spleen after d-amphetamine wash-out (d-amphetamine kept in bath 30 min after maximal contraction had been reached).

Noradrenaline response (NA, 10^{-7} g/ml) was potentiated after d-amphetamine (AMP, 5×10^{-5} g/ml) wash-out at W. Somewhat less potentiation of NA (10^{-7} g/ml) was also observed $1\frac{1}{2}$ hr later when the strip had fully relaxed.

C. Effect of a Subthreshold Dose of d-Amphetamine on Response to Noradrenaline

The above experiments suggested two different effects of d-amphetamine on response to noradrenaline; 1) antagonism at high contraction and 2) potentiation which becomes apparent only after removal of the high dose of d-amphetamine and might be due to a small residual amount of d-amphetamine remaining in the tissue. Small doses of d-amphetamine which were too low to cause a contraction were therefore tested.

Results from preliminary experiments showed a subthreshold dose of d-amphetamine potentiated noradrenaline in cat spleen strips. Experiments were carried out to characterize this property of d-amphetamine.

1) Potentiation of responses to noradrenaline and adrenaline.

Responses to noradrenaline or adrenaline were tested first in the absence and then in the presence of a subthreshold dose of d-amphetamine. At least two reproducible responses to these agonists were recorded before test with d-amphetamine.

10^{-7} g/ml of d-amphetamine was chosen as the subthreshold dose because the threshold dose, giving a contraction of 1-3 mm, varied in the earlier experiments between 3×10^{-7} and 3×10^{-6} g/ml. In a few sensitive strips where 10^{-7} g/ml of d-amphetamine gave a little contraction, a lower dose (3×10^{-8} g/ml) was used.

The results of experiments on strips from normal cats and dogs, reserpine-treated cats and cats with chronically denervated

spleen are summarized in Table 5.

Table 5 shows potentiation of noradrenaline. In 6 other experiments on cat spleens, d-amphetamine (10^{-7} g/ml) increased response to adrenaline (3×10^{-8} or 10^{-7} g/ml) and to noradrenaline (10^{-7} g/ml) to approximately the same extent, 44% and 47% respectively.

2) Duration of potentiation by a subthreshold dose of d-amphetamine.

The potentiation of noradrenaline by the subthreshold dose of d-amphetamine (10^{-7} g/ml) generally did not remain after d-amphetamine was washed out. Responses to noradrenaline (10^{-7} or 3×10^{-7} g/ml) were tested before and in the presence of d-amphetamine (10^{-7} or 3×10^{-8} g/ml) 3 min after its addition to the bath. The fluid was changed and noradrenaline was tested again after 15-60 minutes when the strip had fully relaxed from the preceding test of noradrenaline. In 31 normal cat spleen strips (Table 6) and 6 strips from reserpine-treated cats (Table 7), the subthreshold dose of d-amphetamine increased the response to noradrenaline. In all but two strips from normal cats, noradrenaline responses after removal of d-amphetamine were not potentiated or were slightly reduced. In strips from reserpine-treated cats a little potentiation still remained.

TABLE 5

POTENTIATION OF NORADRENALINE (NA) BY SUBTHRESHOLD DOSE OF d-AMPHETAMINE (AMP) IN SPLEEN STRIPS FROM NORMAL CATS AND DOGS, RESERPINE-TREATED CATS AND CHRONICALLY DENERVATED CAT SPLEENS

	No. of Strips	No. of Animals	Dose of NA (g/ml)	Dose of AMP (g/ml)	% Increase	Mean ±S.E.
Normal Cat Spleen	50	30	10^{-7} or 3×10^{-7}	10^{-7}	20-240	70 ± 5
Normal Dog Spleen	19	8	10^{-7}	10^{-7}	21-106	61 ± 20
Spleen From Reserpine-Treated Cat	11	5	10^{-7} or 3×10^{-7}	10^{-7}	25-116	105 ± 12
Chronically Denervated Cat Spleen	5	3	10^{-7}	10^{-7}	20-100	54 ± 10

TABLE 6

RESPONSES OF CAT SPLEEN TO NORADRENALINE IN THE PRESENCE OF A SUBTHRESHOLD DOSE OF d-AMPHETAMINE AND AFTER d-AMPHETAMINE WASH-OUT

a. Strips from normal cats

Cat No.	Strip No.	Response to Noradrenaline (mm)				% Change in Response *	
		Control	(1) In AMP	(2) After Wash	Time Between (1) and (2)	(1)	(2)
1	1	15	25	20	60 min	+66.6	+33.3
2	1	14	18	13	30 min		
2	2	13.5	26	14	30 min		
2	3	13	24	13	30 min		
2	4	15	23	13	30 min	+67	-3
3	1	8.5	14	11	18 min		
3	2	6	11	7	18 min		
3	3	8	14	9	18 min	+73.4	+20
4	1	26.5	35.5	26	50 min		
4	2	7.5	12	7.5	50 min	+38.1	-1
5	1	22.5	31	17	15 min		
5	2	24	30.5	20	15 min	+32	-22
6	1	19	22.5	19	15 min		
6	2	31	38	30	15 min	+21.2	-2
7	1	9	18	8.5	50 min		
7	2	38	66	34	50 min		
7	3	31	70	29	50 min		
7	4	17	31	16	50 min	+95	-18
8	1	20.5	27	16	40 min		
8	2	23	40	20	40 min	+54.2	-17.4
9	1	15	27	16	15 min	+80	+0.7
10	1	8	19	7	40 min		
10	2	6	16	5	40 min	+115	-14.3
11	1	18	23	17	30 min	+27.8	-0.6
12	1	18	28	12	30 min	+55.5	-33.2
13	1	6	11	4	20 min		
13	2	8	11	6	20 min	+57	-29
14	1	11	22	11	20 min		
14	2	14	23	12.5	20 min	+80	-5.6
15	1	18	39	16	60 min	+116	-1.6
16	1	21	37	18	60 min	+76	-14.3

Dose of noradrenaline used = 10^{-7} or 3×10^{-7} g/ml
Dose of d-amphetamine used = 10^{-7} or 3×10^{-8} g/ml

Change in response was calculated as percentage increase (+) or percentage decrease (-) over the control response.

*In cases where two or more strips were taken from one spleen, their mean responses were used in calculation.

TABLE 7

RESPONSES OF CAT SPLEEN TO NORADRENALINE IN THE PRESENCE OF A SUBTHRESHOLD DOSE OF d-AMPHETAMINE AND AFTER d-AMPHETAMINE WASH-OUT

b. Strips from reserpine-treated cats

Cat No.	Strip No.	Response to Noradrenaline (mm)				% Change in Response *	
		Control	(1) In AMP	(2) After Wash	Time Between (1) and (2)	(1)	(2)
1	1	30.5	78	40	60 min		
1	2	30	87.5	39	60 min	+117.2	+30
2	1	12	20.5	12	40 min		
2	2	13	20	14	40 min	+65	+4
3	1	8	16	8	40 min		
3	2	13	29	16	40 min	+66.5	+19

Dose of noradrenaline used = 10^{-7}
Dose of d-amphetamine used = 10^{-7}

*Mean responses of the two strips from each cat were used for calculation of % change in response.

3) Relationship between dose of d-amphetamine and potentiation.

The threshold dose for d-amphetamine in 4 spleen strips from 4 cats was first found to be 3×10^{-7} g/ml. Noradrenaline (10^{-7} g/ml) was given an hour later to all the strips. When the response to noradrenaline reached its maximum, 4 doses of 3×10^{-8} g/ml d-amphetamine were added cumulatively (5-8 minutes apart) in the baths. The final concentration of d-amphetamine in the bath was 1.2×10^{-7} g/ml which was still subthreshold. The response increased after each addition of d-amphetamine (Fig. 10). The nearer the dose of d-amphetamine to threshold value the greater was the potentiating effect.

Doses of d-amphetamine far below the threshold value (e.g. 10^{-9} g/ml) did not potentiate noradrenaline response. The smallest dose that potentiated noradrenaline (10^{-7} g/ml) in the cat spleen strip was 6×10^{-9} g/ml (15% increase).

4) Loss of effectiveness of repeated potentiating doses of d-amphetamine.

Successive doses of d-amphetamine had less effect in potentiating noradrenaline. Fig. 11 illustrates an experiment with seven successive doses of d-amphetamine. In one strip (time control) d-amphetamine was not used and responses to noradrenaline (10^{-7} g/ml) were tested twenty times over 7 hours (at 15-30 min intervals). In a second strip from the same cat, noradrenaline (10^{-7} g/ml) was tested at the same intervals (15-30 minutes) except that d-amphetamine (10^{-7} g/ml) was given 7 times (30-90 minutes apart) and

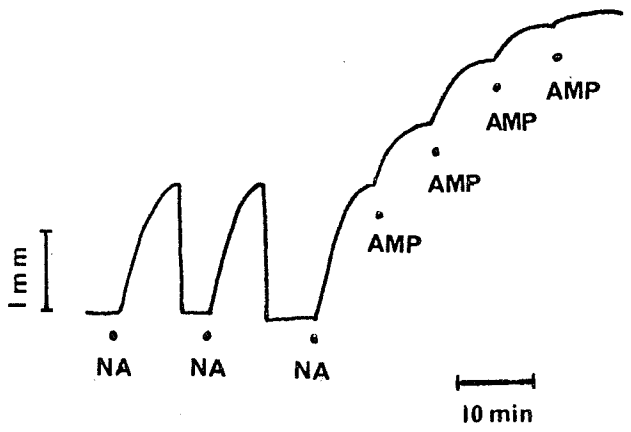


Fig. 10 Increase of noradrenaline response in the presence of increasing subthreshold dose of d-amphetamine.

Noradrenaline (10^{-7} g/ml) was tested at NA. d-Amphetamine (AMP, 3 x 10^{-8} g/ml) was added cumulatively on top of the last NA response.

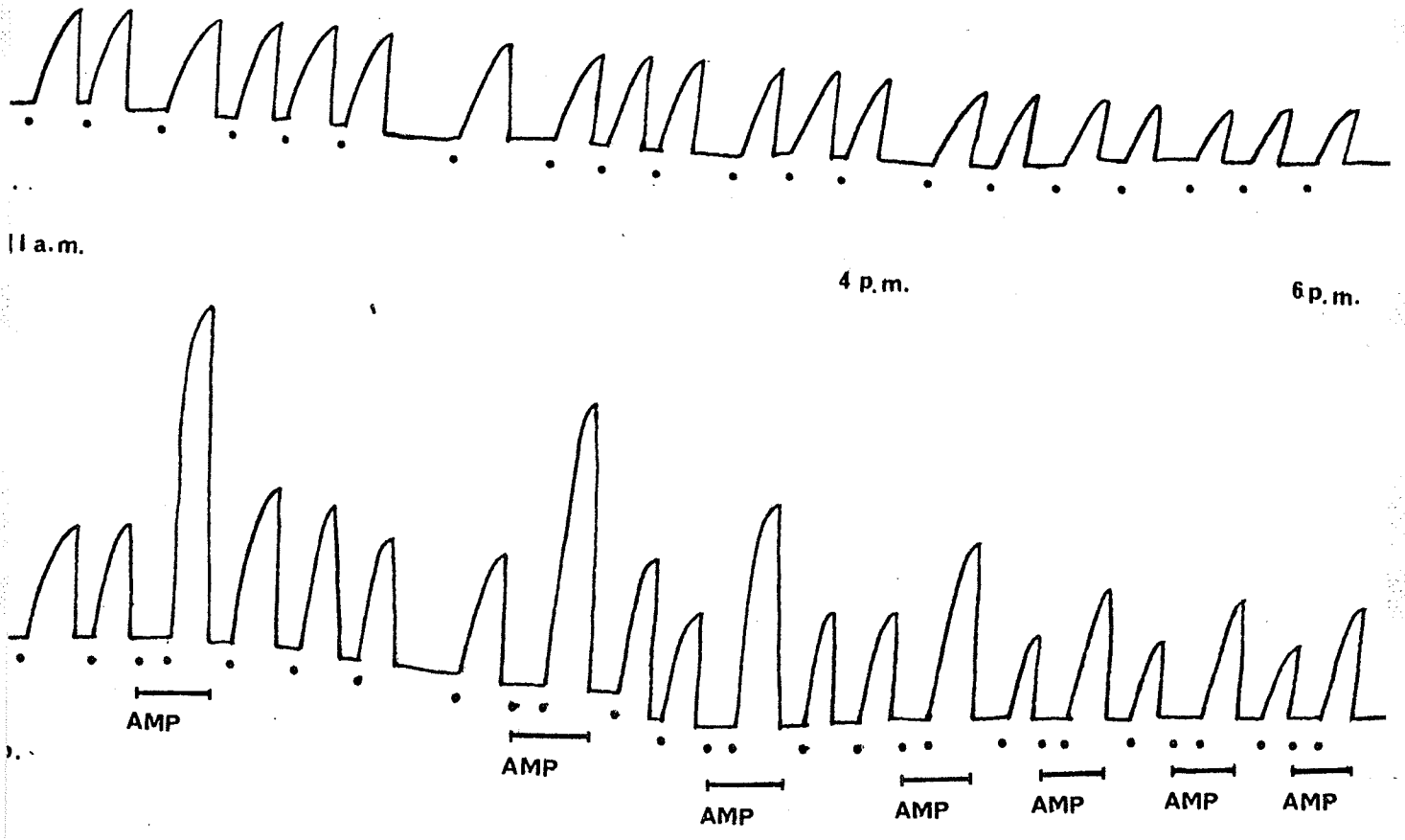


Fig. 11 Loss of effectiveness of repeated potentiating doses of d-amphetamine.

a. Time control strip. Noradrenaline (10^{-7} g/ml), indicated at •) was tested at 15-30 min intervals for 7 hours. The drum was stopped at varying periods after each test.

b. d-Amphetamine tested strip. Individual noradrenaline responses (10^{-7} g/ml, indicated at •) were tested at the same time as in a). d-Amphetamine (10^{-7} g/ml) was added in the bath 7 times 2-3 min before noradrenaline and left in the bath for only the test of a single dose of noradrenaline. ⟵⟶ indicates d-amphetamine left in bath.

left in the bath only for the test of a single dose of noradrenaline. In the time control strip, responses to noradrenaline remained relatively constant over the first 5 hours and gradually reduced in size over the last 2 hours (Fig. 11a). This tendency was also seen in the strip exposed to d-amphetamine (Fig. 11b). The noradrenaline response was potentiated each time d-amphetamine was added in the bath, but the potentiation became less with successive doses of d-amphetamine, the increases being 193, 130, 100, 67, 50, 35 and 35 percent respectively (Fig. 11b).

In five other experiments the potentiating effect of d-amphetamine (10^{-7} g/ml) on noradrenaline (10^{-7} g/ml) was tested twice at an interval of 30 or 60 minutes. The mean increase in the first test was 54.7 ± 8.8%, the second 25.5 ± 2.3%. The difference is statistically significant ($p < 0.05$).

5) Effect of d-amphetamine on dose-response curves of noradrenaline.

Full cumulative dose-response curves on noradrenaline were recorded first without d-amphetamine and later in the presence of a subthreshold dose of d-amphetamine (10^{-7} g/ml). Three spleen strips from 3 cats and 2 strips from 2 reserpine-treated cats were used. Fig. 12 shows the result of a typical experiment on a strip from a normal cat. The greatest potentiation of noradrenaline occurred between doses 10^{-7} - 10^{-5} g/ml noradrenaline. Between these values, the shift of the curve to the left was almost parallel (about $\frac{1}{2}$ log unit). The maximum response was unchanged.

In eight other experiments, the threshold doses of noradren-

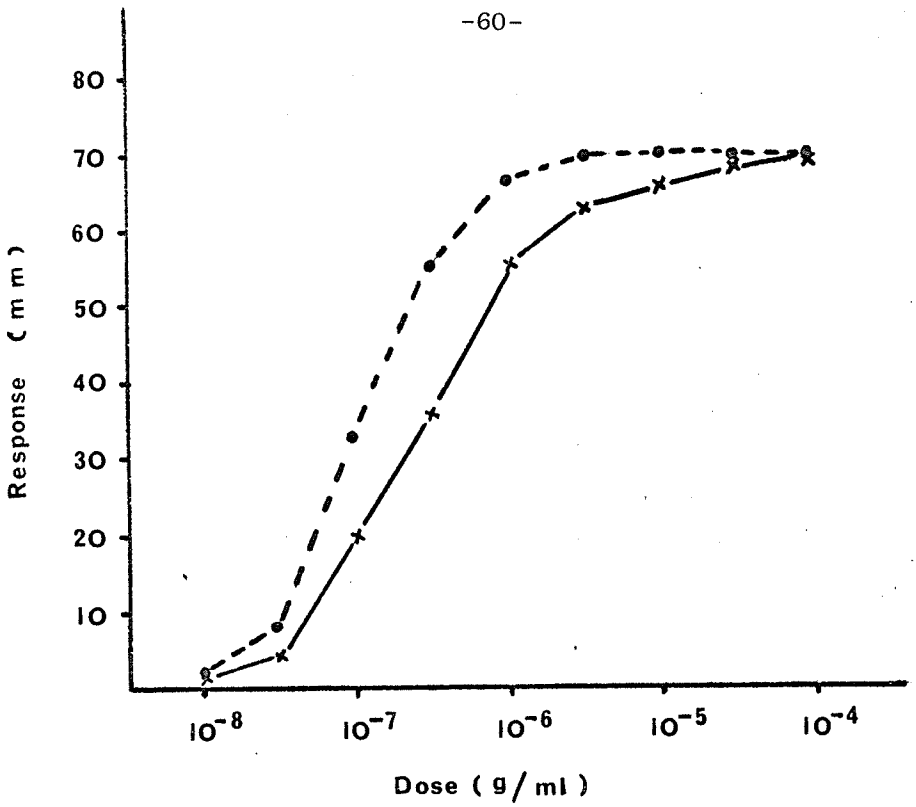


Fig. 12 Cumulative dose-response curves for noradrenaline before and after d-amphetamine (10^{-7} g/ml) in a strip from a normal cat.

×——× before d-amphetamine

●- - -● after d-amphetamine

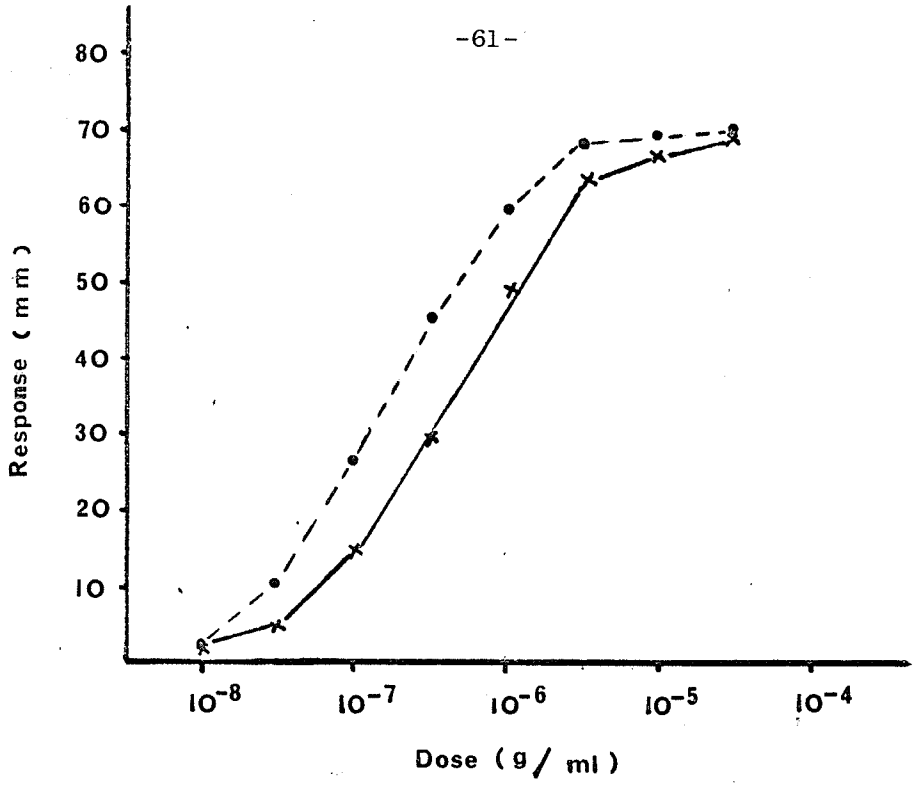


Fig. 13 Cumulative dose-response curves for noradrenaline before and after d-amphetamine (10^{-7} g/ml) in strips from reserpine-treated cats.

Each curve represents the mean of the two strips from 2 reserpine-treated cats.

×——× before d-amphetamine

•– – –• after d-amphetamine

aline causing 1-2 mm contraction were 10^{-8} or 3 x 10^{-9} g/ml. d-Amphetamine (10^{-7} g/ml) did not change the threshold dose. Fig. 13 shows the effect of d-amphetamine on the mean cumulative dose-response curve of 2 strips from 2 reserpine-treated cats. There is a similar pattern of shift to the left after d-amphetamine (10^{-7} g/ml). The maximum and threshold responses were unchanged.

Time control cumulative dose-response curve (in which no d-amphetamine was used) was not done with these experiments. However, experiments done in the same laboratory indicated that shift of the curve was not due to change in sensitivity of the strip with time.

6) Specificity of potentiation.

To see if subthreshold dose of d-amphetamine was specific in potentiating noradrenaline and adrenaline responses, 5 other agonists were tested with subthreshold dose of d-amphetamine. Reproducible responses were first obtained to acetylcholine, bethanechol, histamine, 5-hydroxytryptamine and tyramine in the doses given in Table 8. These doses were chosen so as to give contractions of 10-25 mm, which were expected to lie in the straight part of their dose-response curves. The response to the same dose of agonist was then tested in the presence of a subthreshold dose of d-amphetamine (10^{-7} or 3 x 10^{-7} g/ml). Each agonist was tested in separate strips of spleen, thus avoiding problems of desensitization by testing the strip with two or more agonists.

All the agonists tested were potentiated (Table 8). Potentiation caused by d-amphetamine was thus unspecific in the sense that drugs such as histamine, acetylcholine and bethanechol which have no action on adrenergic receptors, were also potentiated by sub-threshold doses of d-amphetamine.

TABLE 8

POTENTIATION OF ACETYLCHOLINE (Ach), BETHANECHOL (B), HISTAMINE (His), 5-HYDROXYTRYPTAMINE (5-HT) AND TYRAMINE (T) BY SUBTHRESHOLD DOSE OF d-AMPHETAMINE

Agonist	Agonist Concentration	d-Amphetamine Concentration	No. of Strips	No. of Animals	Percentage Increase	Mean ±S.E.
Ach	10^{-7} or 3×10^{-6}	3×10^{-7} or 10^{-7}	6	2 dogs	23-240	83.8 ± 34.8
B	10^{-5}	10^{-7}	6	6 cats	10-118	47 ± 15.5
His	10^{-6}	10^{-7}	9	6 cats	22-133	74 ± 12.6
5-HT	3×10^{-5}	10^{-7}	5	2 cats 1 dog	19-115	63 ± 15.5
T	10^{-6} or 3×10^{-6}	10^{-7}	7	5 cats	13-270	96 ± 40.9

D. Inhibition of Monoamine Oxidase as a Possible Mechanism of Potentiation

Although inhibition of monoamine oxidase has been rejected as the mechanism by which cocaine potentiates noradrenaline (Foster, Ing & Varagic, 1955; Griesmer, Barsky, Dragstedt, Wells & Zeller, 1953) this mechanism is not thereby excluded for d-amphetamine. d-Amphetamine not only is resistant to destruction by monoamine oxidase but also inhibits this enzyme (Blaschko, 1940; Brown & Hey, 1956). The following experiments were done to assess the role of monoamine oxidase inhibition in potentiating responses of spleen strips to noradrenaline.

1) Experiments with nordefrine.

Nordefrine is a sympathomimetic which is not metabolized by monoamine oxidase (Blaschko, Richter & Schlossmann, 1937); hence its potentiation by any drug cannot be due to prevention of its destruction by monoamine oxidase. In experiments done on 6 spleen strips (3 from 2 cats; 3 from a dog) d-amphetamine (10^{-7} g/ml) markedly potentiated nordefrine (3×10^{-7} g/ml) in all strips (Fig. 14). Potentiation varied between 67% and 210% increase, with a mean and S.E. of 151 ± 7.5%.

2) Experiments with iproniazid.

Iproniazid, a well known and potent monoamine oxidase inhibitor (Zeller & Barsky, 1952; Zeller, Barsky, Fouts, Kircheimer & van Order, 1952; Smith, Weissbach & Udenfriend, 1964; Pletscher, 1966)

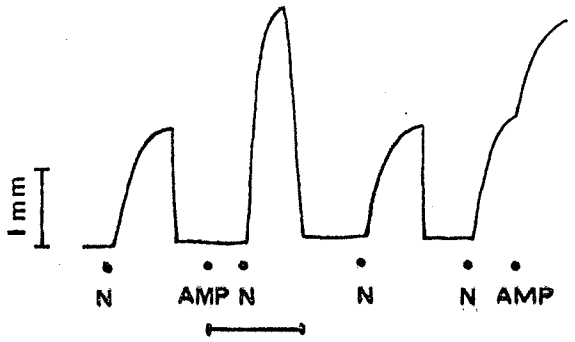


Fig. 14

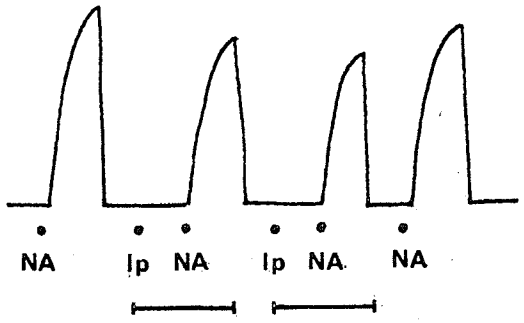


Fig. 15

Fig. 14 Potentiation of nordefrine by d-amphetamine.

A control response to nordefrine (N, 3×10^{-7} g/ml) was recorded. d-Amphetamine (AMP, 10^{-7} g/ml) was added and 3 min later nordefrine (N, 3×10^{-7} g/ml) was tested. Potentiation occurred. Bath fluid was changed and control response to nordefrine was repeated. Subthreshold dose of d-amphetamine (AMP, 10^{-7} g/ml) added when nordefrine response had reached a maximum, caused further contraction.

⊢——⊣ = d-amphetamine kept in bath.

Fig. 15 Failure of iproniazid to potentiate noradrenaline.

The first contraction was a control response to noradrenaline (NA, 10^{-7} g/ml). Iproniazid (Ip, 10^{-7} g/ml) was then added in the bath and 5 min later Na (10^{-7} g/ml) was tested in its presence. There was no potentiation. Bath fluid was changed. Iproniazid (Ip, 10^{-5} g/ml) was added and 5 min later NA (10^{-7} g/ml) was tested again in its presence. There was a little depression. NA (10^{-7} g/ml) tested 40 mins later (iproniazid not in bath) returned to its control heights.

⊢——⊣ = iproniazid kept in bath.

was tested for potentiation on noradrenaline response. Responses of the spleen strips to constant dose of noradrenaline (10^{-7} g/ml) before and after exposure to different doses of iproniazid (10^{-8} - 10^{-5} g/ml) were recorded. Iproniazid was kept in the bath for 3-10 minutes before noradrenaline was added and was not washed out until the strip had responded fully to noradrenaline. No potentiation occurred in any experiment (8 strips from 4 normal cats; 4 strips from 2 reserpine-treated cats) but instead, slight depression was observed in most strips (Fig. 15).

E. Antagonistic Effect of d-Amphetamine and Cocaine

The noradrenaline-potentiating effect of cocaine is well known (Fleckenstein & Bass, 1953; Fleckenstein & Stockle, 1955); Innes & Kosterlitz, 1950; Trendelenburg, 1963, 1966). Although cocaine and d-amphetamine both potentiate noradrenaline, these two drugs exert opposite effects on responses to tyramine. Tainter & Chang (1927), Burn & Tainter (1931) reported depression of tyramine response by cocaine whereas d-amphetamine potentiated tyramine in this investigation (p. 64).

In three experiments cocaine (10^{-6} - 10^{-5} g/ml) and d-amphetamine (10^{-7} g/ml) were tested simultaneously on different strips from the same cats (Fig. 16a, b). In all three experiments cocaine (10^{-6} - 10^{-5} g/ml), added to the bath when tyramine response had come to a maximum, depressed the contraction (30-60%) whereas d-amphetamine (10^{-7} g/ml) potentiated the response to tyramine in the other strips (20-50%). d-Amphetamine (10^{-7} g/ml) added to 3

strips after cocaine had depressed the tyramine response, reversed the depression, bringing the contraction back to its original height (Fig. 16c).

The effect of cocaine (10^{-5} g/ml) on doses of d-amphetamine (10^{-5} - 10^{-4} g/ml) which caused contraction in spleen strips were tested in 5 strips from 3 cats. Cocaine (10^{-5} g/ml) was left in the bath for 3-5 minutes, then d-amphetamine (10^{-5} - 10^{-4} g/ml) was added. No contractions occurred.

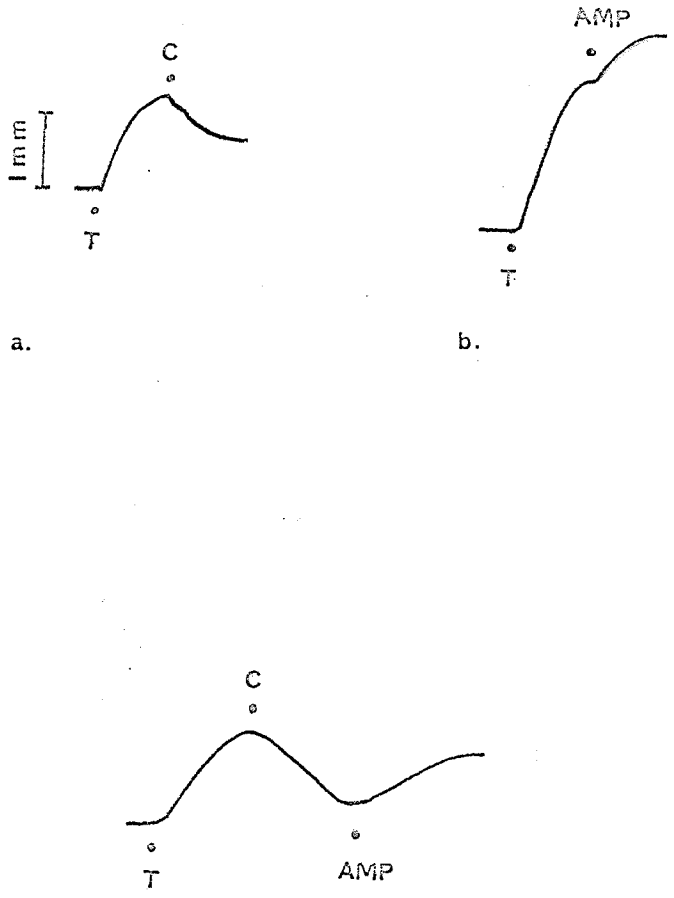


Fig. 16 Effects of cocaine and d-amphetamine on responses to tyramine.

a) Contraction of cat spleen strip to tyramine (T, 10^{-6} g/ml) was depressed by cocaine (C, 10^{-6} g/ml).

b) Contraction of cat spleen strip to tyramine (T, 10^{-6} g/ml) was potentiated by a subthreshold dose of d-amphetamine (AMP, 10^{-7} g/ml).

c) Response to tyramine (T, 10^{-6} g/ml) was first depressed by cocaine (C, 10^{-5} g/ml) and then brought back to its original height by d-amphetamine (AMP, 10^{-7} g/ml).

F. Effect of Congeners of d-Amphetamine

The effect of 20 congeners of d-amphetamine were tested. Their chemical structures are shown in Table 9.

1) Wash-out contraction due to congeners of d-amphetamine.

Methamphetamine, hydroxyamphetamine, phenylethylamine, phentermine and chlorphentermine in concentrations 10^{-5} - 10^{-4} g/ml caused an initial contraction (6-30 mm) and a wash-out contraction (4-30 mm greater than initial) which lasted for 1-3 hours. Ephedrine and mephentermine caused a small wash-out contraction which lasted for only 15-30 minutes. None of the other drugs showed the wash-out contraction typical of d-amphetamine.

2) Potentiating effect.

Effects of subthreshold doses (10^{-8} - 10^{-7} g/ml) of the 20 drugs on responses of the cat spleen to noradrenaline were also tested (Table 9).

a) Phenylpropanolamine, with an additional β-hydroxyl group on the amphetamine structure, also potentiated noradrenaline but potentiation was somewhat less marked than with d-amphetamine.

b) An additional methyl group on amphetamine (methamphetamine) reduced but did not abolish potentiation. Long chain substitution on N (as in prenylamine) resulted in more loss of potentiation; subsensitivity occurred in 2 out of 3 strips tested with prenylamine.

c) Ephedrine, having in addition a β-hydroxyl and an N-methyl group on the amphetamine structure, has less potentiating

activity than compounds with either an additional β-hydroxyl or N-methyl group alone (ephedrine v phenylpropanolamine and methamphetamine); subsensitivity occurred in 4 out of 9 strips.

d) Phentermine and chlorphentermine, with two methyl groups substituted on the α-carbon, still caused potentiation which was equal to that of d-amphetamine. Mephentermine, the N-methylated phentermine, has much less potentiating activity than phentermine or chlorphentermine; subsensitivity occurred in 4 out of 6 strips tested with it.

e) Tyramine (v hydroxyamphetamine) and phenylephrine (v phenylpropanolamine), both lacking an α-methyl group, did not potentiate noradrenaline.

f) Phenylpropylmethylamine, with the methyl group placed on the β-carbon instead of the α-carbon as in methamphetamine, gave less potentiation than methamphetamine.

g) Phenolic hydroxylation at the 4 position (d-amphetamine v hydroxyamphetamine) or chlorination at the 4 position (phentermine v chlorphentermine) did not result in any change of potentiation.

h) The sensitivity of the spleen strips was unchanged by metaraminol, a compound with a phenolic hydroxyl group at the 3 position (v phenylpropanolamine), or by nordefrine with a hydroxyl group on both 3 and 4 positions (v phenylpropanolamine).

i) Methoxylation on either position 2 or positions 2 and 5 (Methoxamine and methoxyphenamine v phenylpropanolamine and methamphetamine) also resulted in lack of potentiation. Methox-

amine caused subsensitivity of the 3 strips tested.

j) Potentiation was unaltered when the aromatic ring of methamphetamine was replaced by a saturated ring (propylhexedrine).

k) Phendimetrazine, with a ring structure substituted for the usual ethylamine in the other drugs, did not cause potentiation; the sensitivity of 3 strips out of 6 was unchanged, while subsensitivity occurred in the other 3.

l) Diethylpropion, besides possessing an α-methyl group and two ethyl groups on N, has an oxygen on the β-carbon. It depressed noradrenaline responses.

m) Phenylethylamine also potentiated noradrenaline.

TABLE 9

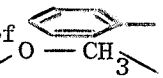
EFFECT OF SYMPATHOMIMETIC AMINES
ON RESPONSE TO NORADRENALINE

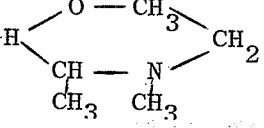
Compounds Tested	Structures	No. of Strips (No. of Cats)	Effect on Response to NA (No. of Strips) % Increase*	% Decrease	Un-changed
d-Amphetamine	H H H CH_3 H	50 (28)	70 ± 5 (50)		
Phenylpropanolamine	H H OH CH_3 H	4 (4)	44.5 ± 6.2 (4)		
Methamphetamine	H H H CH_3 CH_3	8 (6)	47.4 ± 8 (7)		(1)
Ephedrine	H H OH CH_3 CH_3	9 (8)	33.2 ± 5.1.(5)	29.5 ± 8.5 (4)	
Phentermine	H H H $(CH_3)_2$ [1] H	4 (4)	53.8 ± 10.4 (4)		
Chlorphentermine	Cl H H $(CH_3)_2$ [1] H	4 (4)	81.2 ± 10.2 (4)		
Mephentermine	H H H $(CH_3)_2$ [1] CH_3	6 (4)	10, 42 (2)	30.8 ± 4 (4)	
Hydroxy Amphetamine	OH H H CH_3 H	7 (7)	77.9 ± 17 (7)		
Tyramine	OH H H H H	7 (4)		19, 26 (3)	(4)
Phenylephrine	OH H OH H H	3 (3)		16.7 ± 7.7 (3)	
Metaraminol	H OH OH CH_3 H	3 (3)			(3)
Nordefrine	OH OH OH CH_3 H	4 (3)			(4)
Methoxamine	H H [2] OH CH_3 H	3 (3)		23 ± 11 (3)	
Methoxyphenamine	H H [3] H CH_3 CH_3	3 (3)			(3)
Propylhexedrine	4 H CH_3 CH_3	6 (5)	55 ± 10.4 (5)		
Phenylpropylmethylamine	H H CH_3 H CH_3	4 (4)	30, 34 (2)	6 (1)	(1)
Phenylethylamine	H H H H H	4 (4)	55 ± 9.4 (4)		
Phendimetrazine	H H (ring compound) [5]	6 (6)		21.7 ± 4.4 (3)	(3)
Diethylpropion	H H [6] CH_3 [7]	3 (3)		27, 50 (2)	(1)
Prenylamine	H H H CH_3 [8]	3 (3)	20 (1)	5, 10 (2)	

[1] two CH_3 on α-carbon

[2] OCH_3 on position 2 and 5 in the ring

[3] OCH_3 on position 2 in the ring

[4]  instead of 

[5] ring compound = 

[6] $\overset{O}{\parallel}$ on β-carbon

[7] $(C_2H_5)_2$ on the nitrogen

[8] $-CH_2-CH_2-CH$ 

* Mean ± S.E. is given where possible.

DISCUSSION

A. Actions of d-Amphetamine

The results suggest that d-amphetamine has four different actions on smooth muscle of spleen; 1) an indirect action on adrenergic receptors mediated through release of endogenous noradrenaline, abolished by treatment with reserpine; 2) a direct excitatory action not abolished by treatment with reserpine (probably of little importance); 3) supersensitization of tissue to noradrenaline (seen after wash-out and with a low or subthreshold dose of d-amphetamine); 4) antagonism of noradrenaline (seen with an excitatory or high dose of d-amphetamine). A single contraction of the tissue to d-amphetamine therefore represents a resultant effect of all four actions. In such a complicated situation it would be extremely difficult to find out the exact amount of influence contributed by each of the four actions. Our present knowledge about uptake, storage, release and disposal of catecholamines, drug transport across membrane barriers and the cause of supersensitivity is still imcomplete.

The experiments and results will be discussed under various headings and suggestions are made for future experiments whenever it is felt appropriate.

B. Initial and Wash-out Contractions: Significance and Proposed Mechanisms

1) Consistency and duration of wash-out contraction.

Wash-out contraction described here is unique to the spleen strip as opposed to other in vitro smooth muscle preparations such

as the guinea-pig ileum, rabbit aorta, rat stomach strips, etc. It is also a phenomenon seen only with the isomers of amphetamine and several drugs closely resembling amphetamine in structure, such as hydroxyamphetamine, methamphetamine, phentermine, chlorphentermine and propylhexedrine.

The striking characteristic about this wash-out contraction is its long duration and consistency. It was seen in almost every strip of cat or dog spleen tested with an effective dose of d-amphetamine or the drugs mentioned above. Its long duration (1-3 hours) makes it distinguishable from the ordinary wash-out contractions seen quite often with other drugs and on other preparations, such as rabbit aorta, cat papillary muscles, which are of smaller magnitude and last for 3-15 minutes only. These are presumably due to sudden changes in ionic environment surrounding the muscle, bath temperature, or other factors brought about by change of bath fluid. Wash-out contraction caused by removal of d-amphetamine is unlikely to be due simply to sudden change in bath temperature, tension or the ionic composition of bath fluid because it persisted when tension, bath fluid and temperature had obviously become constant again (after 5-15 minutes). In addition, the wash-out contraction still occurred when the bath fluid was changed by overflow instead of emptying and replacement, so that tension changes were minimized. Ohlin & Strömblad (1963) reported that on isolated vas deferens of guinea-pig and rat, not only the addition of noradrenaline, acetylcholine or histamine but also the removal of these drugs from the bath caused contractions which were not due to mechanical

interference and were abolished by the presence in the bath of an antagonist to the drug tested. The authors offered no explanation for this "wash-out effect" they observed. However, these contractions seen after wash-out of drugs lasted for only seconds or minutes. They were single contractions with a sharp spike and occurred with several drugs acting on different receptors. Wash-out contractions seen in the spleen strips differ from this general "wash-out effect" described by Ohlin & Strömblad. They were long-lasting and caused only by d-amphetamine and similar sympathomimetics, and therefore were more specific. Moreover, contractions caused by most agonists on isolated tissue strips do not usually last as long, hence the persistence of the wash-out contraction must carry special meaning.

2) Effect of reserpine treatment.

The results with strips from reserpine-treated animals show that d-amphetamine loses most of its effect on spleen strips lacking noradrenaline stores and that the wash-out contraction depends on intact stores of noradrenaline.

Since the spleen is rich in stores of noradrenaline, Maengwyn-Davies (1965) and her co-workers (1966, 1966a) have used rabbit splenic slices as a functional source of noradrenaline in their experiments. These splenic slices were either untreated or pre-treated with monoamine oxidase inhibitors and were inserted into the organ bath. In accordance with our finding is that l-amphetamine (0.167 µg/ml) failed to cause a contraction of the aortic strips from reserpine-treated rabbits, but after the insertion of frozen or fresh splenic slices from untreated rabbits, l-amphet-

amine caused pronounced contractions (Maengwyn-Davies, Cowan & Koppanyi, 1966; Maengwyn-Davies & Koppanyi, 1966). This clearly indicated the ability of both isomers of amphetamine to release noradrenaline and their dependence on it for their excitatory action.

d-Amphetamine elicited a contraction of only a few millimetres and gave no wash-out contraction in strips from reserpine-treated cats even when the concentration was increased 30-300 times the concentration which was effective in normal strips. Two mechanisms of action of d-amphetamine appear to be involved; 1) a major indirect action through release of noradrenaline which is abolished by treatment with reserpine and 2) a minor direct action which is not abolished by reserpine and which is unlikely to occur except with high doses. Accordingly, wash-out contraction is believed to be the result of persistent or continuous release of endogenous noradrenaline.

Stored noradrenaline was presumably well depleted in the 6 strips of cat spleen and 5 strips of dog spleen used, since a large dose of tyramine (10^{-5} g/ml) failed to contract these strips although the sensitivity of these tissues to noradrenaline was actually increased (p. 34). However, the possible presence of a small store of noradrenaline unaffected by the depleting action of reserpine has been reported by various workers (Kopin & Gordon, 1962; Furchgott, Kirpekar, Rieker & Schwab, 1963; Kopin, 1964; Fischer, Kopin & Axelrod, 1965). Iversen, Glowinski & Axelrod (1965) have also suggested there may be a small store of noradrenaline in tissues after treatment with reserpine, resistant to release by tyramine

but susceptible to release by the ganglionic stimulant, DMPP. If this is so, it then becomes questionable whether the small contraction caused by a very high dose of d-amphetamine was in fact due to a direct action of d-amphetamine on the receptors of smooth muscles, since this small contraction might be due to release of the resistant stores of noradrenaline by d-amphetamine. However, this does not seem probable because an effective or high dose of d-amphetamine strongly antagonizes noradrenaline (p. 37). Receptor protection experiments showed that d-amphetamine (10^{-4} g/ml) although it failed to contract spleen strips from reserpine-treated cats, prevented phenoxybenzamine from blocking noradrenaline but not histamine. This provides evidence that d-amphetamine combines with the adrenergic receptors. Innes (1962) reported that 5-hydroxytryptamine acted on adrenaline receptors in splenic smooth muscle. It appears likely that amphetamine, 5-hydroxytryptamine and noradrenaline act on common receptors in the spleen, i.e. besides release of noradrenaline, amphetamine has a direct affinity for these common receptors.

The observation that d-amphetamine antagonizes noradrenaline also supports the assumption that d-amphetamine has a direct action on the adrenergic receptors. This would lead to the conclusion that d-amphetamine has a very low intrinsic activity after combination with the receptors.

3) Antagonism of noradrenaline by d-amphetamine.

The results indicate that d-amphetamine antagonizes noradrenaline in strips from normal and reserpine-treated cats. Thus d-amphetamine acts as a partial agonist on the adrenergic receptors.

Results on reserpine-treated preparations have special significance since reserpine depletes not only noradrenaline but also 5-hydroxytryptamine stores in spleen and other peripheral and central sites (Pletscher, Shore, Brodie, 1955, 1956; Erspamer, 1956; Garattini & Valzelli, 1965), and release of 5-hydroxytryptamine by d-amphetamine was not unknown (Paasonen & Vogt, 1956). These results would indicate that antagonism of noradrenaline by d-amphetamine was not due to release of 5-hydroxytryptamine which presumably has a lower intrinsic activity than noradrenaline. According to Innes (1962), 5-hydroxytryptamine has a direct action on adrenergic receptors on spleen strips and this direct action can be observed only with large doses when its indirect mechanisms of noradrenaline release have been inactivated by cocaine or previous treatment with reserpine. Contractions due to 5-hydroxytryptamine 5×10^{-3} g/ml in cocaine or reserpine-treated preparation were generally smaller than contractions due to one fiftieth or one-hundredth of this dose in strips from normal cats, thus giving strong support to the view that 5-hydroxytryptamine is a partial agonist.

The antagonism by d-amphetamine is quickly reversible, since removal of d-amphetamine permits full action of noradrenaline on the receptors and hence appearance of wash-out contraction. Depression of the wash-out contraction by d-amphetamine reintroduced into the bath suggests that the wash-out contraction is partly due to removal of the antagonistic effect of d-amphetamine. Since there is evidence that wash-out contraction is probably due to release of endogenous noradrenaline it is indicated that d-amphetamine

antagonizes the noradrenaline it releases.

4) 5-Hydroxytryptamine on wash-out contraction.

Wash-out contractions were depressed by 5-hydroxytryptamine but not by histamine and acetylcholine. This agrees well with the concept that 5-hydroxytryptamine is a partial agonist (Innes, 1962) if, as concluded above, the wash-out contraction is due to noradrenaline release. As with d-amphetamine, 5-hydroxytryptamine appears to have a low intrinsic activity on the adrenergic receptors.

Since the conclusion that wash-out contraction is due to release of noradrenaline is based on indirect evidence, direct evidence is desirable. This could be obtained by collecting the bathing medium, preferably by superfusion, and analysing the medium for noradrenaline by chemical method; this is believed to be feasible since the sensitivity of the fluorometric analysis for noradrenaline is now on the nanogram level (Goldstein, Friedhoff & Simmons, 1959; Häggendal, 1966).

5) Relationship between onset of wash-out contraction and time of exposure to d-amphetamine.

A direct relationship between onset of wash-out contraction and time of exposure of tissue to d-amphetamine has been observed (p. 31). These experiments also show an inverse relationship between time of exposure and the size of wash-out contraction. Long exposure to d-amphetamine may release most of the noradrenaline in the stores so that little is left to be released. Turnover rate of noradrenaline at these sites may also be slowed, since d-amphetamine inhibits *in vitro* the enzyme responsible for the

final step in the biosynthesis of noradrenaline, dopamine-beta-hydroxylase (Goldstein & Contfera, 1961; Goldstein, Anagnoste, Lauber & McKerenghan, 1964). Maengwyn-Davies et al, (1966) had observed that the noradrenaline stores in the spleen could be exhausted by d- or l-amphetamine. Edge (1964) reported observations on the guinea pig vas deferens which parallel ours. In concentrations from 1 to 100 g/ml amphetamine potentiated responses to hypogastric nerve stimulation. A concentration of 500 g/ml of amphetamine had a blocking action which was "rapidly reversed on washing out, the responses then showing the persistent potentiation regularly seen after washing following lower concentrations of the drug". Two possible causes of block were considered by the author, prevention of release by high doses of amphetamine or antagonism of noradrenaline since high concentrations of amphetamine also antagonized the action of noradrenaline added to the bath. Similarly, prevention of release of noradrenaline by high doses of amphetamine remains a possibility in spleen strips.

Another aspect to be considered is the release mechanism. Wash-out contraction is sustained even if the strip is washed many times. The mechanism through which d-amphetamine does this is therefore puzzling. It has been postulated that tyramine stoichiometrically releases noradrenaline from the isolated storage granules of the heart and splenic nerves by a displacement mechanism (von Euler & Lishajko, 1960; Schümann & Philippu, 1961). Amphetamine has also been reported to act in the same way (Burn, 1965), but the mechanism and kinetics of release of noradrenaline by d-amphet-

amine on the spleen have not yet been so extensively studied. Whereas tyramine is highly sensitive to the action of monoamine oxidase, d-amphetamine has an advantage over tyramine in the study of kinetics of noradrenaline release since it resists monoamine oxidase and prevents destruction of noradrenaline by this enzyme. Instead of displacing noradrenaline mole for mole from the storage sites, d-amphetamine might impair the storage mechanism and result in continuous release of noradrenaline. Also, it might affect the amine concentration mechanism, the combination of amines to ATP, or the turnover rate of noradrenaline in the storage granules.

The possibility that blockade of inactivation of the released noradrenaline may be responsible for the long duration of the wash-out contraction should also be considered. d-Amphetamine has several actions, some of which like blockade of noradrenaline uptake (Burgen & Iversen, 1965) and inhibition of monoamine oxidase, may well affect the disposal of the noradrenaline it releases. Although monoamine oxidase does not play a major role in inactivating circulating catecholamines (Axelrod, 1959), this neuronal enzyme still takes an important part in the metabolism of endogenous catecholamines (Shore, 1962; Kopin, 1964). Shore (1962) pointed out that blockade of monoamine oxidase is an important factor in preventing depletion of catecholamines by reserpine since catecholamines released by reserpine are mainly destroyed by monoamine oxidase.

Amphetamine blocks or impairs uptake of noradrenaline at uptake sites. Iversen (1963, 1965a, b) reported two distinct processes of accumulating noradrenaline in the rat heart, $uptake_1$ and $uptake_2$

which operates at different perfusion concentrations of noradrenaline. Both d- and l-amphetamine have been found to inhibit uptake$_1$ and uptake$_2$ (Iversen, 1964; Burgen & Iversen, 1965). Fluorescence microscopy showed that preincubation of tissue with d-amphetamine decreased the number and intensity of the very fine catecholamine-containing (mainly noradrenaline) terminals on the peripheral adrenergic nerves (Malmfors, 1965) and in the brain (Carlsson, Lindquist, Dahlström, Fuxe & Masuoka, 1965). These authors postulated that amphetamine causes release of catecholamines from the storage granules as well as extragranular sites and that one of the sites of action of amphetamine is on the "membrane pump" (Malmfors, 1965; Carlsson & Waldeck, 1965, 1966) which presumably operates to concentrate noradrenaline within the nerve axons and terminals.

Since d-amphetamine has been found to inhibit monoamine oxidase (see p. 65) and block uptake and restorage of noradrenaline ("cocaine-like" agent, as described by Trendelenburg, 1966), the major disposition of noradrenaline released would be through destruction by catechol-O-methyl-transferase and leaking into the bathing medium. Preincubation of the spleen strips with catechol-O-methyl-transferase inhibitor may therefore significantly prolong the wash-out contraction or increase leakage of intact noradrenaline into the medium.

C. Potentiation of Noradrenaline (Supersensitivity)

1) After wash-out of effective doses of d-amphetamine.

Strips from both normal and reserpine-treated animals were

supersensitive to noradrenaline after d-amphetamine wash-out. This is especially interesting in strips from reserpine-treated cats because these strips were already more sensitive to noradrenaline than normal strips, and the increase in response was not small (increasing by 38-200%) and lasted more than an hour after wash-out. Potentiation of noradrenaline response occurred after d-amphetamine wash-out in the normal strips exposed to d-amphetamine for 5-15 min, just enough time for contraction to attain maximum, or for 30-40 min after contraction had attained maximum. Supersensitivity appears to be due to part of the administered d-amphetamine remaining in the tissue after wash-out, since a small subthreshold dose of d-amphetamine caused supersensitivity to noradrenaline in strips from both normal and reserpine-treated animals.

Maengwyn-Davies et al (1966) also observed that, after a few effective (on normal aortic strips) or ineffective (on aortic strips from reserpine-treated rabbits) doses of l-amphetamine (levedrine), responses to the directly acting sympathomimetic amine, phenylephrine, increased. The authors believe this "suggests that residual levedrine remained in the contractile tissue even after thorough washing. This residual levedrine may have occupied less specific binding sites (silent receptors) and thus increased the concentration of phenylephrine available for attachment to the specific (active) receptor in the effector organ". The enhancement of response to noradrenaline seen after d-amphetamine wash-out is explainable in terms of blockade of uptake sites if residual amounts of d-amphetamine still remain in the tissue and if affinity of d-amphetamine

for the unspecific uptake site is great.

2) Subthreshold dose of d-amphetamine.

A subthreshold dose of d-amphetamine makes the spleen strips significantly more sensitive to noradrenaline (Table 5). This occurred not only in strips from normal cats and dogs but also in strips from reserpine-treated cats and chronically denervated cat spleens, which were already supersensitive to noradrenaline (p. 53; Karr, 1966). In addition, the increase in response is significantly greater in strips from reserpine-treated cats. It is tempting to speculate that supersensitivity caused by reserpine and d-amphetamine arise from two different mechanisms. Karr (1966) suggested that reserpine causes supersensitivity by a mechanism involving postreceptor events which are response-limiting, and, if d-amphetamine causes supersensitivity by mechanisms at the prereceptor or receptor levels, then the effects of the two drugs together could be additive. However, our data do not provide definitive evidence on the mechanism involved.

Supersensitivity caused by a subthreshold dose of d-amphetamine is in several ways qualitatively different from denervation supersensitivity and cocaine-induced supersensitivity, the most obvious being loss of effectiveness of repeated potentiating doses of d-amphetamine, independence of presence of noradrenaline stores and sensitization to both directly and indirectly acting sympathomimetics. These characteristics of d-amphetamine-caused supersensitivity are discussed in the following.

a) Loss of effectiveness of repeated potentiating doses.

Tachyphylaxis of response to d-amphetamine has been reported by numerous workers (Alles, 1933; Winder et al., 1948; Hanna, 1960, 1960a; Cowan et al., 1961; Maengwyn-Davies et al., 1966) but whether it extends to the potentiating action of d-amphetamine is doubtful, since the basic mechanisms of tachyphylaxis are no more understood than is the cause of supersensitivity. It has been suggested that d-amphetamine tachyphylaxis may be due to reduction of noradrenaline available for release and that full responses can be restored after resting and washing the tissue when there is no interference with noradrenaline synthesis (Maengwyn-Davies et al., 1966). However, we have observed a greater potentiation of noradrenaline response in spleen strips lacking noradrenaline stores (from reserpine-treated cats), therefore release does not seem to play an important role in causing this supersensitivity; hence reduced release of endogenous noradrenaline cannot account for the loss of effectiveness with repeated potentiating doses of d-amphetamine. However, this loss of effectiveness with successive potentiating doses of d-amphetamine has been shown only in strips from normal cats. Experiments on strips from reserpine-treated cats have not been done.

b) Independence of noradrenaline stores.

Supersensitivity caused by d-amphetamine does not seem to depend on the integrity of the noradrenaline stores. Responses to noradrenaline are further enhanced by d-amphetamine in strips, which lacking noradrenaline stores, are already supersensitive to noradrenaline (after reserpine or chronic denervation) and which,

according to the "accommodation hypothesis" have presumably lost their "accommodation" to noradrenaline. Therefore, supersensitivity here does not seem explainable on the basis of the "accommodation hypothesis" or the uptake hypothesis (p. 20) since the major uptake sites being the nerve endings, have already been eliminated by chronic denervation.

c) Sensitization to indirectly acting sympathomimetic amine - tyramine.

The noradrenaline-releasing action of tyramine is now well established (Burn & Rand, 1958; Lockett & Eakins, 1960; von Euler & Lishajko, 1960; Schümann & Philippu, 1961; Lindmar & Muscholl, 1961; Potter et al., 1962; Kuntzman & Jacobson, 1964; Gutman & Weil-Malherbe, 1966).

Cocaine, although it potentiates tissue responses to noradrenaline, antagonizes the action of tyramine (Tainter & Chang, 1927; Burn & Tainter, 1931) and other indirectly acting sympathomimetics by preventing them from releasing noradrenaline (Burn & Rand, 1958; Lockett & Eakins, 1960; Trendelenburg, 1961; Lindmar & Muscholl, 1961). Our results show that the effect of a subthreshold dose of d-amphetamine on the response of tyramine is distinctly different from that of cocaine. Whereas cocaine depresses the response to tyramine in cat spleen, d-amphetamine potentiated it (p. 65). Furthermore, a subthreshold dose of d-amphetamine reverses the cocaine-induced depression of the response to tyramine in these strips. An antagonism may exist between cocaine and d-amphetamine but our present data are too scanty to support this possibility.

However, cocaine did prevent usually effective dose of d-amphetamine from causing a contraction of the cat spleen. This is probably due to interference of noradrenaline release.

d) Inhibition of monoamine oxidase.

Two types of experiments suggest that d-amphetamine does not cause supersensitivity by inhibiting monoamine oxidase. The subthreshold dose of d-amphetamine markedly potentiated responses of cat and dog spleen strips to nordefrine, a sympathomimetic which is not a substrate for monoamine oxidase; also iproniazid, a monoamine oxidase inhibitor, did not potentiate noradrenaline (p. 67). Since the formation of an active intermediate of iproniazid may be required for maximal inhibition (Davison, 1957; Zeller et al., 1958; Kory & Mingioli, 1964), the precaution was taken to give a longer exposure time (up to 20 min). However, regardless of the dose of iproniazid (10^{-8} - 10^{-5} g/ml) or exposure time (3-20 min) potentiation did not occur, but instead slight depression was observed. This agrees with the observations of Tsai & Fleming (1965) that iproniazid and other monoamine oxidase inhibitors unspecifically antagonized the actions of noradrenaline, acetylcholine and potassium in the isolated nictitating membrane of the cat. Noradrenaline was antagonized by iproniazid added acutely to the isolated preparation, as well as in preparations from cats pretreated with iproniazid for 20-24 hours. In view of the possible masking of other effects by this unspecific antagonism, it is still possible that monoamine oxidase may play a small role in causing supersensitivity.

e) Specificity.

The subthreshold dose of d-amphetamine also potentiated responses of the spleen strips to bethanechol, acetylcholine and histamine (p. 64), which act on different receptors from those for adrenaline and noradrenaline; thus supersensitivity caused by the subthreshold dose of d-amphetamine seems rather unspecific. Responses to other agonists which act on the adrenergic receptors (either directly or indirectly through noradrenaline release) like 5-hydroxytryptamine, tyramine and nordefrine are also potentiated. Cocaine has also been reported by various workers to potentiate the action of acetylcholine (Rosenblueth, 1932; Thompson, 1958; Koppanyi & Feeney, 1959). However, Trendelenburg (1962, 1963) found that on cat nictitating membrane cocaine shifted only the lower 1/3 of the dose-response curve of acetylcholine to the left, apparently not due to a true sensitization of the membrane by cocaine but to an additive effect of endogenous noradrenaline released by cocaine. In our experiments 'potentiation' of acetylcholine and histamine responses might be due to an additive effect of noradrenaline released by d-amphetamine in an amount insufficient to cause a contraction by itself. Lack of data on strips from reserpine-treated animals and on shift of dose-response curves renders this question inconclusive. It would also be desirable to see if a subthreshold dose of d-amphetamine potentiated contractions due to potassium and barium since specificity is an important factor to consider in the study of cause of supersensitivity. The current uptake hypothesis does not explain the unspecificity of supersensitivity caused by decentral-

ization, denervation and reserpine treatment (Cannon, 1939; Fleming, 1963; Schmidt & Fleming, 1964; Trendelenburg & Weiner, 1962) for unspecificity suggests changes beyond the receptors.

It is not suprising that no hypothesis can yet explain the complexity of supersensitivity, since different types of supersensitivity may be produced by different procedures or agents, and different mechanisms are likely to be involved. Although some workers (Maxwell et al., 1959; Maxwell, Wastila & Eckhardt, 1966; Karr, 1966) do not accept the uptake hypothesis as the explanation for cocaine-induced supersensitivity, this does not necessarily include d-amphetamine-caused supersensitivity. Profound changes in drug concentration and inactivation could be brought by a combination of the actions of d-amphetamine: on noradrenaline release (perhaps including displacement of noradrenaline from unspecific tissue uptake sites), on monoamine oxidase (not necessarily inhibition but its attachment and resistance to the enzyme), and on blockade of uptake of noradrenaline (uptake by nerves and unspecific tissue uptake). These actions may be complementary to each other and exert an effect which is not seen when each action is being analysed separately. Moreover, the importance of each action may vary with the kind of drug used or preparation used. For example, release of noradrenaline and action on monoamine oxidase may be more important in causing potentiation of tyramine response than noradrenaline response. Prevention of replenishment of empty stores would seem much more important than release in strips from reserpine-treated animals.

It is well understood that other possibilities such as changes in conformation of receptors and changes in postreceptor events may be involved. However, an analysis of the underlying causes of supersensitivity induced by d-amphetamine is beyond the scope of this thesis.

D. Effects of Congeners of d-Amphetamine

1) Initial and wash-out contraction.

Typical initial and wash-out contractions occurred only with some compounds very closely related to d-amphetamine in structures and all these compounds possess strong central nervous system or anorexiant effects. It seems likely that they affect the stores of noradrenaline and other adrenergic innervated structures through the same mechanisms as d-amphetamine does.

2) Potentiation of noradrenaline by subthreshold doses.

Observations were made on whether certain modifications of the d-amphetamine structures would retain or bring about loss of potentiating property in the hope of determining the structural requirement of compounds producing such a potentiation. From the effects of the 20 drugs tested, the following conclusions are arrived at.

a) Compounds lacking an α-methyl group (tyramine v hydroxyamphetamine; phenylephrine v phenylpropanolamine) do not potentiate noradrenaline, thus an α-methyl group seems to be an essential moity.

b) Substitution of a second methyl group on the α-carbon does not abolish potentiation (phentermine and chlorphentermine).

c) Either β-hydroxylation (d-amphetamine v phenyl-

propanolamine) or N-methylation alone (d-amphetamine v methamphetamine) reduces but does not abolish potentiation. β-Hydroxylation and N-methylation together brings about greater loss of potentiation (ephedrine v phenylpropanolamine and methamphetamine). These two groups may cause some steric hindrance on the α-methyl group. When two methyl groups are already on the α-carbon, an additional N-methyl group gives the same effect as that seen with ephedrine.

d) Long chain substitution on N (as in prenylamine) results in great loss of potentiation.

e) β-Methylation instead of α-methylation (phenylpropylmethylamine v methamphetamine) results in decreased potentiation. Again the methyl group on the α-position is important.

f) Potentiation remains intact with phenolic hydroxylation or chlorination at the 4 position (d-amphetamine v hydroxyamphetamine, phentermine v chlorphentermine), but phenolic hydroxylation at the 3 position (metaraminol v phenylpropanolamine) or at both 3 and 4 positions (nordefrine v phenylpropanolamine) abolishes potentiation.

g) Methoxylation on either position 2 or positions 2 and 5 (methoxamine and methoxyphenamine v phenylpropanolamine and methamphetamine) also abolishes potentiation.

h) Substitution of the aromatic ring with a saturated ring does not alter the potentiating property (propylhexedrine v methamphetamine).

i) Diethylpropion, a compound with two ethyl groups on N and an oxygen on β-carbon crowding around the α-methyl group, depresses instead of potentiating noradrenaline responses.

j) The α-methyl group seems to play an essential role in potentiation but, as in so many studies of structure-activity relationships, there is an exception, phenylethylamine, which does not possess an α-methyl group yet potentiates noradrenaline responses.

SUMMARY

1. In spleen strips from cats and dogs d-amphetamine (10^{-5} - 10^{-4} g/ml) caused initial contractions which took 5-15 min to reach their maximum of 5-35 mm. Wash-out of d-amphetamine caused a further contraction which reached 10-40 mm in 15-30 min, then remained constant for 1-2 hr before relaxation began. Relaxation took another 30-40 min. Contractions from d-amphetamine left in the bath for 30-70 min did not reach the height of the wash-out contraction.

2. Increased exposure to d-amphetamine delayed the onset of and reduced the size of the wash-out contraction. No wash-out contraction occurred after prolonged exposure to d-amphetamine (30-40 min after maximum contraction was reached).

3. d-Amphetamine (10^{-4} - 10^{-5} g/ml) caused neither an initial nor a wash-out contraction in strips from reserpine-treated cats and dogs, indicating that the major action of d-amphetamine was through release of noradrenaline. In several strips of cat spleen higher doses of d-amphetamine (3×10^{-4} - 10^{-3} g/ml) caused 1-3 mm contractions but no wash-out contraction. Receptor protection experiments showed d-amphetamine protected responses to noradrenaline but not histamine against the action of phenoxybenzamine in strips from reserpine-treated cats, providing evidence that d-amphetamine combined directly with adrenergic receptors.

4. d-Amphetamine ($1 - 5 \times 10^{-5}$ g/ml) reduced the responses to noradrenaline (10^{-7} g/ml) to about 1/10 in strips from both

normal and reserpine-treated cats. d-Amphetamine (10^{-5} g/ml) reintroduced into the bath after wash-out depressed the wash-out contraction was due to continued release of noradrenaline and the removal of the antagonistic effect of d-amphetamine.

5. 5-Hydroxytryptamine also depressed the wash-out contraction, while other agonists, such as acetylcholine, histamine and noradrenaline, did not. 5-Hydroxytryptamine and d-amphetamine were believed to act as partial agonists in antagonizing the action of endogenous noradrenaline, which presumably caused the wash-out contraction.

6. Spleen strips from reserpine-treated and normal cats and dogs were more sensitive (38-260% increase) to noradrenaline (10^{-7} g/ml) after wash-out of d-amphetamine (10^{-5} - 3×10^{-4} g/ml). This was believed to be due to residual amounts of d-amphetamine left in the tissue.

7. A subthreshold dose of d-amphetamine (10^{-7} or 3×10^{-8} g/ml, threshold being 3×10^{-7} - 3×10^{-6} g/ml) potentiated responses to noradrenaline (10^{-7} g/ml or 3×10^{-7} g/ml) in strips from normal cats and dogs, reserpine-treated cats and cats with chronically denervated spleens. Potentiation in strips from reserpine-treated cats was greatest. Responses of normal cat spleen strips to adrenaline (3×10^{-8} - 10^{-7} g/ml) were also potentiated by d-amphetamine (10^{-7} g/ml); the increase of responses to adrenaline and noradrenaline seemed approximately the same. The cumulative dose-response curves for noradrenaline in the presence of d-amphetamine (10^{-7} g/ml) were shifted almost

in parallel to the left for $\frac{1}{2}$ a log unit (in strips from both normal and reserpine-treated cats).

8. Enhancement of noradrenaline ($1 - 3 \times 10^{-7}$ g/ml) disappeared within 15-60 min after wash-out of the subthreshold dose of d-amphetamine in strips from normal cats. In strips from reserpine-treated cats a little potentiation still remained after 40-60 min.

9. The degree of potentiation increased as the dose of d-amphetamine was increased towards threshold. The lowest dose of d-amphetamine which potentiated noradrenaline (10^{-7} g/ml) was 6×10^{-9} g/ml (15% increase).

10. Successive doses of d-amphetamine had less effect in potentiating noradrenaline. The increase in noradrenaline response (10^{-7} g/ml) due to a second subthreshold dose of d-amphetamine (10^{-7} g/ml), 30-60 min after the first, was significantly less than the increase due to the first dose. In one experiment noradrenaline (10^{-7} g/ml) was tested 7 times with d-amphetamine (10^{-7} g/ml). The potentiation of noradrenaline became less with successive doses of d-amphetamine, the increases being 193, 130, 100, 67, 50, 35 and 35 per cent respectively.

11. The subthreshold dose of d-amphetamine also potentiated histamine, acetylcholine, bethanechol, 5-hydroxytryptamine, and tyramine. Thus potentiation was unspecific in the sense that drugs such as histamine, acetylcholine and bethanecol which have no action on adrenergic receptors were also potentiated.

12. Potentiation caused by the subthreshold dose of d-amphetamine

did not seem explainable by inhibition of monoamine oxidase, since nordefrine (3×10^{-7} g/ml) which is not metabolized by monoamine oxidase was greatly potentiated by a subthreshold dose of d-amphetamine (10^{-7} g/ml) and large doses of iproniazid, a monoamine oxidase inhibitor, did not potentiate noradrenaline in strips from reserpine-treated or normal cats; instead it caused slight depression.

13. There seems to be an antagonistic effect between d-amphetamine and cocaine. Whereas cocaine (10^{-6} - 10^{-5} g/ml) depressed responses to tyramine (10^{-6} g/ml), d-amphetamine (10^{-7} g/ml) caused potentiation. d-Amphetamine (10^{-7} g/ml), added to the bath after cocaine (10^{-6} - 10^{-5} g/ml) had depressed the responses to tyramine, reversed the depression, bringing the contraction back to its original height. In the presence of cocaine (10^{-5} g/ml) the usual excitatory doses of d-amphetamine (10^{-5} - 10^{-4} g/ml) did not cause any contraction in cat spleen strips.

14. Among the 20 sympathomimetics tested, only a few caused the characteristic initial and wash-out contractions described for d-amphetamine; these were l-amphetamine, hydroxyamphetamine, methamphetamine, phentermine, chlorphentermine, propylhexedrine and phenylethylamine.

15. The effects of subthreshold doses of the 20 sympathomimetics on responses to noradrenaline in normal cat spleen strips were tested and observations were made on whether certain changes in the amphetamine structure brought about retention or loss

of potentiation.

It was concluded that d-amphetamine has four actions on the smooth muscle of spleen; 1) a major indirect action mediated through release of stores of noradrenaline, 2) a minor direct action which is not abolished by reserpine-treatment and is produced only by large doses, 3) supersensitization to noradrenaline, seen after wash-out of excitatory doses or in the presence of subthreshold doses of d-amphetamine, 4) antagonism of noradrenaline, seen with excitatory or high doses of d-amphetamine.

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