STUDIES OF SUPERSENSITIVITY IN SMOOTH MUSCLE

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William James Davidson

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ABSTRACT

The purpose of this study was to characterize and determine possible mechanisms responsible for supersensitivity produced by cocaine, denervation and reserpine in isolated cat spleen strips and to compare these results with those obtained with guinea-pig ileum and rat uterus. Cat spleen strips were used because they provide a preparation which contains smooth muscle which contracts when activated by various biogenic amines. The cat spleen also has a high endogenous noradrenaline content and is therefore suitable for the study of effects of cocaine, denervation and reserpine on catecholamine uptake; the currently popular hypothesis to explain supersensitivity is that the concentration of a catecholamine in the extracellular fluid immediately in contact with the alpha adrenergic receptors is normally limited by uptake into the adrenergic nerves and that procedures which reduce uptake will therefore cause supersensitivity. However, this hypothesis does not provide a satisfactory explanation for potentiation of agonists which act on other receptors.

Experiments on the uptake of isoprenaline and noradrenaline in cat spleen are reported. Cocaine potentiated isoprenaline and noradrenaline in spleen strips from normal cats and from cats pretreated with reserpine. The experiments with denervation of cat spleen showed that the major sites of uptake of isoprenaline and noradrenaline are in the adrenergic nerves and although isoprenaline was taken up almost as well as noradrenaline by spleen strips from cats treated with reserpine, cocaine blocked uptake of noradrenaline but did not reduce uptake of isoprenaline. It is concluded that inhibition of uptake is not the

mechanism by which cocaine potentiates the effect of isoprenaline on the spleen and might only be a contributory factor in the case of noradrenaline potentiation.

Cocaine did not appear to change the affinity of the receptors, since it did not alter the pA_3 values of phentolamine against noradrenaline and histamine. The finding that cat spleen has no adrenergic receptor reserve allows comparisons of affinity to be made between different sympathomimetic amines by a second technique, competitive receptor protection. Although values obtained are in good agreement with predicted values, caution is advised in the use of this technique in other than normal tissues, because changes in affinity of phenoxybenzamine, the non-equilibrium blocking agent used, could lead to erroneous conclusions.

Cocaine can specifically potentiate responses to catecholamines in cat spleen and rat uterus or produce an unspecific supersensitivity, whereas denervation of cat spleen and rat uterus gives an unspecific supersensitivity. Cocaine and denervation have no effect on maximum responses to agonists. Reserpine also produces an unspecific supersensitivity; responses to all agonists tested on ileum, spleen and uterus are potentiated. Reserpine causes the maximum responses to all agonists tested to increase. No change in adrenergic receptor reserve could be detected after cocaine, denervation or reserpine and because reserpine increases maximum responses to agonists in guinea-pig ileum where there already is a receptor reserve, it is suggested that reserpine acts at a post-receptor level on factors which normally limit the maximum contraction.

The unspecificity of supersensitivity produced by cocaine, denervation and reserpine suggests that they do so by mechanisms which

are independent of their effects on catecholamine uptake. Because denervation and reserpine require time (1 - 14 days) to produce supersensitivity it is suggested that alteration of normal mediator influences causes synthesis of proteins with structural differences which produces supersensitivity. Cocaine produces supersensitivity in a few seconds and it is suggested that supersensitivity produced by cocaine can be explained by changes in permeability of the smooth muscle cells in the effector organ. New approaches to determine whether any of the suggested mechanisms are correct are described in the discussion.

To my parents,

my beloved wife and children,

Norah, Ross, Linda and Morag,

and my dear friend, Roddie.

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INTRODUCTION AND STATEMENT OF THE PROBLEM

The terms "potentiation", "synergism", and "supersensitivity" have been used in different senses by various authors to describe enhanced responses of an excitable tissue to stimuli; such terms give no indication of what the underlying mechanisms may be, and will be used here with no attempt to imply a knowledge of such mechanisms.

The subject of supersensitivity has been extensively reviewed from time to time (Cannon and Rosenblueth, 1949; Furchgott, 1955; Emmelin, 1961; Trendelenburg, 1963 and 1966), and various hypotheses have been put forward to account for observed supersensitivity; though in general these have not withstood the test of critical experimentation.

This section will review some important procedures and drugs that produce supersensitivity and some concepts, in connection with proposed mechanisms of supersensitivity.

Budge (1855) first reported that cat iris responded to asphyxia by "paradoxical dilatation" after its sympathetic nerve supply had degenerated and later Lewandowsky (1899) reported chronically denervated cat nictitating membrane responded to adrenal extracts with increased magnitude. In a review by Cannon and Rosenblueth (1949) it is apparent that initial observations of supersensitivity were first associated with surgical denervation of smooth or striated muscle but it is now known that many if not all excitable tissues may be made supersensitive by interruption of the normal innervation or treatment with various pharmacological agents. For example chronic administration of phenoxybenzamine caused supersensitivity of cat nictitating membrane,

(Nickerson and House, 1958); cocaine potentiates responses of nictitating membrane of the cat to nerve stimulation (Rosenblueth and Rioch, 1933; Innes and Kosterlitz, 1954a); reserpine has been reported to produce supersensitivity in a variety of tissues (Innes, 1960; Schmidt and Fleming, 1963; Brutsaert, 1964).

Cannon (1939) suggested that as supersensitivity produced by denervation was unspecific an explanation might be permeability changes to stimulating substances in such denervated tissues. Although most procedures cause an unspecific supersensitivity, i.e. responses to many types of stimuli are enhanced; to produce a general field theory many hypotheses have ignored this lack of specificity and concentrated on mechanisms which would explain increased responses to catecholamines. The more pertinent hypotheses will be reviewed in chronological order.

ENZYME INHIBITION

Blaschko et al. (1937) showed that extracts of various tissues caused the oxidation of adrenaline and postulated that an amine oxidase in their extracts was responsible.

Burn (1952) suggested that potentiation caused by cocaine was due to inhibition of monoamine oxidase (MAO) and Burn and Robinson (1953) reported that denervation supersensitivity could be correlated with the fall in MAO levels in muscle. They proposed that supersensitivity to adrenergic stimuli produced by denervation or cocaine was a result of MAO inhibition.

This hypothesis could not explain later findings that more potent inhibitors of MAO than cocaine failed to potentiate responses to catecholamines (Griesemer et al., 1953; Furchgott, 1955; Varagić, 1956).

Furchgott (1955) suggested that another enzyme was responsible for reducing the meta-hydroxyl group of adrenaline and noradrenaline and that this enzyme could be inhibited by cocaine and explain the observed supersensitivity.

Axelrod (1957) identified this enzyme catechol-O-methyl transferase (COMT) as an important enzyme in the normal destruction of catecholamines. Though some inhibitors of COMT could cause a slight increase in catecholamine responses (Bacq et al., 1959; Crout, 1961) this could not explain supersensitivity produced by cocaine or denervation, since cocaine or denervation did not inhibit COMT (Wylie et al., 1960; Missala, 1966) and Potter et al. (1965) showed that O-methylation of catecholamine was actually increased after denervation.

REDUCED ACCOMMODATION OF EFFECTOR CELLS

Fleckenstein and Bass (1953), realizing both denervation and cocaine reduced the normal continuous discharge of noradrenaline from postganglionic nerve fibres, suggested that the normally released transmitter keeps effector cells of smooth muscle in a state of accommodation or subsensitivity. Removal of normal transmitter function reduced this accommodation and increased sensitivity of the effector cells. Burn and Rand (1959), abandoning the "Enzyme Hypothesis", pointed out that reserpine as well as denervation resulted in a loss of tissue stores of catecholamines. They agreed with Fleckenstein and Bass (1953) that the normal continuous release of noradrenaline might well keep the sensitivity of effector cells low and that removal of this released transmitter inhibition would result in supersensitivity.

Emmelin (1961) and Trendelenberg and Weiner (1962) also favoured

this interpretation with an emphasis on inactivity of the end organ as the important factor. This hypothesis did not take into account that cocaine does not abolish release of transmitter from nerve endings and in fact cocaine potentiates responses of nictitating membrane to sympathetic nerve stimulation (Rosenblueth and Rioch, 1933; Innes and Kosterlitz, 1954a; Trendelenberg, 1959). Cervoni and Kirpekar (1966) showed prolonged infusion of catecholamines did not affect responses of decentralized nictitating membrane to subsequent doses of catecholamines. Kirpekar, Cervoni and Furchgott (1962) showed the increase in sensitivity to exogenous noradrenaline in nictitating membrane was not related to the decrease in stores of noradrenaline in the membrane.

Recently interest has been renewed in this hypothesis by the work of Barnett et al. (1968) who showed cocaine increased the maximum response of isolated vas deferens to noradrenaline and postulated potentiation was due to prevention of a desensitizing process of auto-inhibition. The work of Fleming and colleagues (1968) shows that in guinea-pig ileum chronic ganglion blockade produced an unspecific supersensitivity whereas chronic treatment with reserpine produced a specific supersensitivity in that responses to ganglion stimulating agents were potentiated whereas stimulants acting on effector cells were not. They attributed this to the fact that, although reserpine depleted the ileum of catecholamines, acetylcholine content was increased. They conclude that unspecific supersensitivity is caused by prolonged interruption of normal stimulatory pathways to a smooth muscle producing changes in smooth muscle probably beyond the receptors.

DEFORMATION OF RECEPTORS

Clark (1937) suggested that cocaine changed the cell receptor "in some manner so that either the rate of association of adrenaline is increased or its rate of dissociation is decreased". Since then Maxwell and co-workers (1958, 1959, 1965) have suggested deformation of receptors by combination of sensitizing agents with allosteric sites could be a cause of supersensitivity. Karr (1966), finding that pre-exposure to various adrenergic agents prevented or reduced the potentiating effect of cocaine in cat spleen whereas other agents acting on cholinergic or histaminergic receptors did not, suggested allosteric transitions affecting affinity of the alpha adrenergic receptor.

Green and Fleming (1967) determined pA_2 and pD_2 values in the nictitating membrane of the spinal cat and found chronic denervation, chronic decentralization, chronic reserpine, and acute treatment with cocaine did not alter pA_2 values for noradrenaline-phentolamine whereas all but cocaine significantly lowered pD_2 values for noradrenaline-phenoxybenzamine. In contrast these authors (1968) did not find any significant alteration in pA_2 or pD_2 values by the same treatments in similar experiments on cat spleen. However these authors failed to produce supersensitivity in cat spleen by reserpine treatment and their denervation procedure only was partially successful. Consequently comparisons between their two studies are not really meaningful. In the first study where all procedures used did produce supersensitivity they conclude that the action of cocaine is entirely presynaptic whereas decentralization, denervation and reserpine treatment exert at least part of their action postsynaptically.

UPTAKE HYPOTHESIS

The currently popular hypothesis is that the concentration of catecholamine in the extracellular fluid immediately in contact with the adrenergic receptors is normally limited by uptake into the adrenergic nerves, and supersensitivity to adrenergic stimuli is due to impairment or blockade of tissue catecholamine uptake, thereby increasing the concentration near receptors.

Trendelenburg (1959) reconfirmed that in the spinal cat cocaine potentiated responses of the blood pressure to noradrenaline and responses of nictitating membrane to preganglionic nerve stimulation and noradrenaline. Analysis showed there was a decreased rate of removal and inactivation of noradrenaline from plasma. He suggested cocaine potentiated noradrenaline responses by delaying its activation, either limiting cellular permeability to noradrenaline or binding of noradrenaline to the cell. Macmillan (1959) also suggested supersensitivity produced by cocaine might be explained by prevention of uptake of noradrenaline into tissue stores.

In 1961 Whitby et al. showed uptake of ^{3}H-noradrenaline was highest in tissues with a rich sympathetic innervation, e.g. heart, spleen and adrenals, and in the same year this group of workers also showed that the uptake of ^{3}H-noradrenaline in a tissue was greatly reduced after denervation (Hertting et al., 1961a). This observation has been confirmed by many workers (Strömblad and Nickerson, 1961; Hertting and Schiefthaler, 1964; Potter et al., 1965; Iversen, 1965). Further evidence in support of locallization of the major stores of noradrenaline in adrenergic neurones came when Falk and co-workers (1962, 1964),

Dahlstrom and Fuxe, 1964 and Corrodi and Hillarp (1963, 1964) perfected the technique of Eränko (1955) of visualizing catecholamines by a histochemical technique.

Malmfors (1965), using this technique in the iris of rats depleted of noradrenaline stores by pretreatment with reserpine, showed that exogeneous noradrenaline was taken up by such tissues in adrenergic nerve fibres and a similar study by de la Lande et al. (1967) of the central artery of the rabbit ear produced the same findings.

Evidence that cocaine inhibited uptake of noradrenaline into adrenergic nerve fibres was quickly forthcoming (Hillarp and Malmfors, 1965; Malmfors, 1965) and numerous authors using either ^{3}H-noradrenaline in untreated tissues or unlabelled noradrenaline in tissues depleted of catecholamine stores by reserpine have confirmed these findings, e.g. (Whitby et al., 1960; Hertting et al., 1961; Furchgott et al., 1963; Gillespie and Kirpekar, 1965).

Many observations indicating that inhibition of noradrenaline uptake runs parallel with potentiation fit well with this hypothesis to the extent that Iversen (1967) has stated potentiation of catecholamines is a property which all inhibitors of uptake may be expected to share.

The uptake hypothesis is still currently popular with many workers who choose to ignore various anomalies that exist and whose experiments are in many cases designed to support but never to attack this concept. Even if interference with uptake of catecholamines accounted for supersensitivity to catecholamines such inhibition does not explain supersensitivity to agonists which act on other receptors, e.g. acetylcholine and histamine (Rosenblueth, 1932).

To expand this argument we first note that, because the effect of reducing uptake of catecholamine should be an increased concentration of amine available at the receptor site, responses to catecholamines should be potentiated whether mediated via alpha or beta receptors. However, potentiation of catecholamines at beta receptors is inconsistent. Burn and Tainter (1931) found cocaine reduced responses to adrenaline in virgin cat uterus where beta receptors are preponderant. Innes and Kosterlitz (1950) showed cocaine and denervation increased chronotropic responses to noradrenaline in cat heart but did not alter responses to adrenaline.

Euler (1938) showed cocaine potentiated responses to catecholamines in isolated human placenta, a preparation which has little or no innervation and as stated above (page 6) major sites of storage and uptake are in adrenergic neurones. Bevan and Verity (1967) removed the adventitial layer from rabbit aortic strips and though subsequent histological examination showed that strips were nerve free, cocaine still potentiated responses to adrenaline. Maxwell et al. (1966) showed that in rabbit aorta cocaine could inhibit uptake of noradrenaline up to 30% before any potentiation was observed.

Kalsner and Nickerson (1969) developed an elegant technique of oil immersion to study inactivation of various biogenic amines in strips of rabbit aorta and potentiation produced by cocaine. They clearly showed inactivation could not explain the observed supersensitivity, and postulated a direct action of cocaine on effector cells.

While interference of uptake might have explained supersensitivity to catecholamines it is not a satisfactory explanation for unspecific

supersensitivity observed after various procedures, e.g. denervation, reserpine pretreatment. There is even disagreement on whether one treatment does or does not produce supersensitivity to a given stimulus tested on the same organ in the same species, e.g. cocaine, acetylcholine and cat nictitating membrane (Rosenblueth, 1932; Tsai et al., 1968).

RECEPTOR CONCEPT

Effect of a given drug on a given effector system, such as activation of smooth muscle with subsequent contraction or relaxation is usually interpreted in terms of an interaction between the drug and a specific receptive substance or receptor of the cells of the effector system.

The idea of a specific receptive substance as a site of action for drugs, such as nicotine and curare in the myoneural junction was introduced by Langley (1905).

It is not possible to give an exact definition of the term receptor; because receptors have not yet been identified, and therefore it has not been possible to study the primary physical or chemical change which occurs when a drug and receptors interact. The term receptor, therefore has different connotations and there is no unanimity of opinion among biologists.

In this study, the term receptor will be used to indicate the postulated specific molecular sites on structures in or on an effector cell with which molecules of a specific agonist must react in order to elicit the characteristic response of the cell to the agonist and exclude various types of binding sites included under the term drug "Acceptor" (Fastier, 1964).

Current theories of the mechanism of action of drugs at receptor level is based primarily on the classical work of Clark (1926, 1933, 1937) and Gaddum (1926, 1937) who showed that drug receptor interactions closely approximate the relationships encompassed by the Langmuir (1918) adsorption isotherm which is similar to the Michaelis-Menten equation. Clark (1937) proposed the equilibrium theory, according to which the drug-receptor interactions are governed by the laws of mass action. In its simplest form Clark's theory involves the assumption that the effect is proportional to the number of receptors occupied by the drug, i.e. maximal response occurs when all receptors are occupied. In order to explain competitive antagonism, Clark hypothesized that both agonist and antagonist compete for the same receptors and that the effect is directly proportional to the amount of agonist which is combined with receptors.

More recent work has revealed several facts which cannot be explained by this theory in its simplest form, and has thrown doubts on Clark's assumption regarding proportionality between effect and receptor occupancy. Some of these facts are as follows: 1) the slope of the log-dose-effect curve is often significantly different from that predicted by theory; 2) some of the compounds in a series of analogues are weak agonists, yet antagonize the effects of a strong agonist. Quantitative studies with such compounds and consideration of the above mentioned anomalies led to the concept of intrinsic activity (Ariens, 1954) and efficacy (Stephenson, 1956). Stephenson (1953, 1956) pointed out that the effect of an agonist depends not only on its affinity for the receptors, but also on its ability to produce an effect when combined. This

idea was developed independently by Ariens (1954) who spoke of the affinity and the intrinsic activity of drugs.

According to these modifications of Clark's theory, a drug may antagonize other drugs by occupying nearly all the receptors and yet produce a small effect itself -- a 'partial agonist' or a drug with 'dualism in action'.

Nickerson (1956) and Stephenson (1956) also showed that Clark's original assumption that 100% receptor occupancy was necessary to obtain the maximum response to any agonist did not hold true in guinea-pig ileum and independently introduced the concept of a receptor reserve or spare receptors.

Paton (1961) introduced an elegant theory of mechanisms of drug action. It is based on the idea that drugs act on receptors only at the moment of combination. He suggested that many drug action phenomena, e.g. competitive antagonism and desensitization could be explained on the assumption that the stimulant effect of a drug depends, not on the number of occupied receptors but on their rate of occupation. He thus assigned a crucial role to the dissociation constant of a drug and has some evidence to indicate that it is this property of drugs which determines their agonistic and antagonistic activity (Paton, 1961; Paton and Waud, 1962, 1964).

Belleau (1964) pointed out that all theories so far proposed, whether modifications of Clark's classical theory or entirely new concepts, suffer from the absence of a biophysical basis and do not provide a qualitative interpretation of drug-properties at the receptor level. Consequently he advanced a molecular theory, called Macromolecular

Perturbation Theory. He elaborated this theory for the muscarinic cholinergic receptors and suggested that the muscarinic receptor is acetylated acetylcholinesterase.

All receptor theories have been found inadequate to explain fully the mechanism of all types of drug action. Doubtless further theories will be propounded as more work is done in the field. This present work will be discussed only in terms of the concept of occupation of receptors, with the response dependent upon affinity and intrinsic activity.

The complexity of supersensitivity has not been adequately explained by any of the existing hypotheses. This study is concerned with supersensitivity to biogenic amines but is confined to studies on smooth muscle, the major part of the study being done on cat spleen. Studies on other organs were done to determine if any major feature of supersensitivity produced by a certain procedure, e.g. denervation, would hold true between organs and species.

There is general agreement on much of the sequence of events in excitation-contraction coupling in skeletal muscle and to a lesser extent in cardiac muscle. Excitation-contraction coupling in smooth muscle, though widely investigated, is still poorly understood (Somlyo and Somlyo, 1968). Research on interaction of drugs with tissue receptors is still in its infancy, albeit a prolonged childhood (Ehrenpreis, Fleisch and Mettag, 1969).

Many pharmacological agents can produce supersensitivity and this study was limited to two agents, cocaine and reserpine. (a) Cocaine whose ability to cause supersensitivity was first reported by Fröhlich

and Loewi (1910) but whose mechanisms of action were still described by Furchgott (1963) as the "cocaine paradox". Cocaine has the advantage of producing supersensitivity when given acutely in vivo or in vitro.
(b) Reserpine depletes tissue stores of catecholamines and produces supersensitivity in various organs and species, though a controversy exists as to time of treatment required to produce supersensitivity and specificity of such supersensitivity. (Innes, 1960; Trendelenburg and Weiner, 1962; Schmidt and Fleming, 1963; Green and Fleming, 1968; Kalsner and Nickerson, 1969b).

The other procedure for producing supersensitivity was the classical technique of denervation. In this thesis denervation refers to interruption of postganglionic nerve fibres to an effector organ as opposed to decentralization which is interruption of preganglionic nerve fibres.

SELECTION OF THE EXPERIMENTAL TISSUES

To study mechanisms of action of such procedures on excitation-contraction all experiments reported have been done on isolated tissues. These have the advantages over whole animal experiments in that reflex effects are eliminated, more accurate predictions of actual drug concentration at a site of action can be made, administration of drug is not limited by side effects, e.g. blood pressure, full dose response curves to a given agonist can usually be obtained, and enough strips can usually be obtained from a single organ to enable appropriate control experiments to be done at the same time.

As this study was to clearly delineate effects produced by the various stated procedures on a particular organ in a chosen species

and then determine to what extent these findings would hold true in different organs in other species, and finally to determine if any mechanisms of actions of these procedures could be uncovered it was essential the main experimental tissue satisfy certain basic requirements.

The organ chosen should contain smooth muscle which

a) should respond to various stimuli with a reasonable sensitivity;

b) cocaine, reserpine and denervation should produce supersensitivity when stimuli are tested in the in vitro preparation;

c) to study the effects of interference of catecholamine storage and uptake, it should have an adrenergic supply.

The organ chosen to satisfy these criteria was cat spleen. The use of "exsected spleen" was first described by Sherrington (1919) in a manual of practical exercises in physiology. Federicq (1929) reported that adrenaline contracted isolated dog spleen and Vairel (1933) stated that this held true for isolated splenic capsule preparations of dog, rabbit, tench and frog. Saad (1935) extended these observations to include man, cat, guinea-pig, rat and buffalo.

Innes (1962) renewed popularity in this preparation when he reported 5-hydroxytryptamine (5-HT) and adrenaline acted on the same receptors, that reserpine depleted the catecholamine content of spleen, cocaine potentiated responses to catecholamines and acetylcholine and histamine each acted on its own specific receptors. Bickerton et al. (1962) used isolated cat spleen strips to study adrenergic blocking agents and in 1963 Bickerton reported isoprenaline contracted cat spleen by an action on alpha adrenergic receptors.

Euler (1956) reported spleen had a rich adrenergic innervation

and contained high stores of catecholamines, while Karr (1966) showed denervation of the cat spleen produced supersensitivity when tested in an isolated preparation. Since this work was started Fillenz (1970) has reported on innervation of the cat spleen with a full histochemical and electromicroscopy analysis and shows that the main splenic artery has both a cholinergic and adrenergic nerve supply, but the spleen itself only contains adrenergic nerve fibres. The smooth muscle is organized in a number of different ways. (a) Capsule and avascular trabeculae with loosely packed smooth muscle cells embedded in connective tissue. (b) Arteries with a muscular media consisting of tightly packed smooth muscle cells containing no collagenous tissue. (c) Veins with no proper wall but are endothelial-lined channels in smooth muscle trabeculae. Tight junctions are seen between smooth muscle cells.

Histochemical analysis showed all the adrenergic nerve fibres are related to smooth muscle cells but there are considerably fewer nerve bundles than smooth muscle cells. None of the histological techniques used showed any ganglion cells beyond the bifurcation of the main splenic artery. Two types, "large and small" granular vesicles were seen in adrenergic fibres, both types being present in terminal fibres but only large granular vesicles in non terminal axons. Fillenz suggests large vesicles are concerned with synthesis and small vesicles with release of transmitter.

Rat uterus and guinea-pig ileum were chosen for comparison with cat spleen. In rat uterus the predominant adrenergic receptor is beta "inhibitory", though alpha "stimulatory" receptors also are present (Brooks et al., 1965). This tissue was therefore different from cat

spleen where the alpha receptor is dominant but is sensitive to a variety of stimulating agents. Surgical denervation of rat uterus is also possible. In guinea-pig ileum also beta "inhibitory" receptors are dominant, but alpha receptors are inhibitory (Kosterlitz and Watt, 1964). This preparation is sensitive to a variety of stimulating agents, and therefore unspecific supersensitivity can be studied on it. Although a vast amount of work has been done on this preparation, it has been used little if at all for the study of mechanisms of supersensitivity.

SECTION II

METHODS

A. CAT SPLEEN

General Procedures

Cats of either sex weighing 0.5 - 3.8 kg were fasted overnight and killed by a blow on the head. The spleen was removed as quickly as possible and placed in Krebs-Henseleit solution at 4°C. Dissection was done on a filter paper placed on an inverted Petri dish, saturated with the bathing solution. Strips 20 mm long and 3 mm wide were carefully cut from the edge of the spleen. Each strip was attached by a loop of undyed terylene thread to a glass hook which was then placed in an organ bath. The strip was suspended vertically and attached by a second terylene thread to a light Palmer frontal writing lever.

The organ bath contained about 10 ml of bathing solution at $37° \pm 0.2°C$ and was drained and filled through openings at the bottom of the chamber. In some experiments an overflow wash procedure was used to change the bathing fluid. Strips were allowed to equilibrate for at least one hour before tests were started. During this time, the bathing fluid was changed every 10 minutes.

Isotonic contractions against 1 g tension were recorded on a kymograph at 6.5 times magnification and 1 mm/min paper speed.

B. GUINEA-PIG ILEUM

General Procedures

Guinea-pigs of either sex weighing 250 - 500 g were fasted overnight and killed by a blow on the head. Only non-terminal segments of the ileum, taken at least 10 cm. from the ileocecal junction (Munro, 1952) were used. Mesenteric attachments were removed and sections 25 mm

in length were suspended in 10 ml organ baths containing Krebs-Henseleit solution at 37° ± 0.2°C.

Isotonic contractions against 1 g tension were recorded on a kymograph at 6.5 times magnification and 1 mm/min paper speed. Tissues were suspended for at least an hour before tests began. The bathing fluid was changed every 10 minutes during the period.

C. RAT UTERUS

General Procedures

Mature virgin female rats weighing 220 - 350 g were fasted overnight and killed by a blow on the head. Both uterine horns were quickly removed and placed in a modified Dale's solution at 4°C. Mesometrial attachments were removed and segments of either uterine horn 20 mm in length were suspended in 10 ml organ baths containing the modified Dale's solution at 28° ± 0.2°C.

Isotonic contractions against 1 g tension were recorded on a kymograph at 6.5 times magnification and 1 mm/min paper speed. Tissues were suspended for at least an hour before tests began. The bathing fluid was changed every 10 minutes during this period.

EXPERIMENTAL PROCEDURES

I. TREATMENT OF ANIMALS WITH RESERPINE

Animals to be depleted of noradrenaline stores were given reserpine 1.0 mg/kg intraperitoneally twenty four hours before an experiment. Tissues were tested for depletion by assay for catecholamine content.

II. DENERVATION OF THE CAT SPLEEN

Postganglionic denervation of the spleen was done by removal of the celiac ganglion. Anesthesia was induced with pentobarbital sodium (25 mg/kg) and maintained with open drop ether. All operative procedures were done under aseptic conditions and 1 ml of Fortamycin (Ayerst) given intramuscularly. The animals were allowed to recover in a heated cage.

III. DENERVATION OF THE RAT UTERUS

Denervation of the uterus was done by sectioning the least splanchnic nerves. Anesthesia was induced by chloralose-urethane, 1%-10% (2.5 ml/kg; 50% of usual anesthetic dose) and maintained with open drop ether. All operative procedures were done under aseptic conditions and the animals allowed to recover in a heated cage.

IV. DOSE-RESPONSE CURVES

The spleen was first repeatedly exposed to a low concentration (approximately ED_{25}) of the agonist being tested till a constant response was obtained. Dose-response curves were then determined by tests of graded increases in the concentration of agonist, with cumulative additions of agonist. Each addition was made only after a plateau response

to the previous dose had been reached. The concentration added each time was the difference between the existing bath concentration and the desired concentration and any inactivation of drug in the bathing fluid was ignored. Drug concentrations were increased until no further increase in response occurred. The bathing fluid was then replaced with fluid containing no drug and changed every 5 minutes until the response had returned to the original baseline, when the tissue was considered ready for further tests.

With guinea-pig ileum and rat uterus, responses of the tissues to the various agonists were tested at intervals of 10 minutes. Each dose of the agonist remained in the bath until the full contraction for that dose was obtained, usually less than 2 minutes after the drug was added. The bath was then emptied and the fluid replaced. The fluid was exchanged at 5 minute intervals between drug additions.

V. DETERMINATION OF pA_3

Schild (1947) defined pA_x as the negative log to the base 10 of the molar concentration of an antagonist which will reduce the effect of a multiple dose (x fold) of an active drug to that of a unit dose. To estimate the pA_x it is necessary to expose pieces of tissue to various concentrations of the antagonist and to estimate in each case whether the response to the multiple dose x is greater or less than the required value. pA_x values vary with the time for which the tissues have been exposed to the antagonist. Hence it is essential to give the exposure time when pA_x values are being reported.

A modified version of the method of Schild was used. A unit dose of agonist, e.g. 10^{-7} g/ml noradrenaline is tested till a constant

response is obtained. Then the multiple dose 3×10^{-7} g/ml noradrenaline is tested till the response is constant. The unit dose response is then retested to ensure that desensitization is not occurring. With phentolamine as the antagonist, a dose 10^{-10} g/ml is added to the bath 5 minutes before the multiple dose of agonist is again tested. The tissue is then washed until the noradrenaline response is gone and the procedure repeated with increasing concentrations of phentolamine, one log unit increase at a time, until the multiple dose response of the agonist is less than the original unit dose response. The results obtained are then plotted as a graph and the actual pA_3 value can be calculated. This value can be quickly checked on fresh preparations. The tissue is then washed and checked to see if the original agonist response can be obtained, to show that the antagonism was reversible. The pA_x value obtained is expressed as a molar concentration of antagonist.

VI. ESTIMATION OF AFFINITY OF SYMPATHOMIMETICS BY RECEPTOR PROTECTION

Selective receptor protection was used by Furchgott (1954) to distinguish between the acetylcholine, adrenaline, histamine and 5-hydroxytryptamine receptors of smooth muscle in rabbit aorta. He used large concentrations of individual agonists for selective protection of receptors against blockade by Dibenamine, a non-equilibrium antagonist. We used a similar procedure with phenoxybenzamine as the non-equilibrium antagonist to estimate affinity of various sympathomimetics in cat spleen.

Four strips from a single spleen were mounted as described in the general procedure method section. Cumulative dose-response curves were obtained to adrenaline in each strip. Three of the four strips were exposed to a concentration of 10^{-4} g/ml of different sympathomimetic

amines, the fourth strip received no agonist. Adrenaline was used as one of the sympathomimetic amines in every experiment. After 5 minutes exposure to each agonist and without washing, phenoxybenzamine 2.5 x 10^{-7} g/ml was added to each of the four baths for the next 5 minutes. The drugs were then washed out and washes repeated every 5 minutes until the record of the contraction due to the agonist had returned to the original baseline. This generally took sixty to ninety minutes. Cumulative dose-response curves to adrenaline were then repeated in each strip; the maximum responses before and after phenoxybenzamine were compared, and the results expressed as a percentage of the control

VII. DETERMINATION OF SPARE RECEPTORS IN CAT SPLEEN

Nickerson (1956) demonstrated that the guinea-pig ileum had a receptor reserve (spare receptors) for histamine. Stephenson (1956), using a different approach, also pointed out that the guinea-pig ileum had spare receptors so that a 100% occupancy of receptors was not necessary to obtain the maximum response to an agonist. A consequence of this concept is that the response to a drug for which there are spare receptors will not be proportional to receptor occupancy throughout the whole dose range.

We modified the technique of Nickerson to determine the receptor reserve to sympathomimetic amines in cat spleen. Four strips from a single spleen were used. A cumulative dose-response curve to a single sympathomimetic amine was obtained in each strip. Three of the four strips were exposed to a single concentration of phenoxybenzamine, 10^{-10}, 10^{-9}, 10^{-8} g/ml respectively, for 5 minutes. The fourth strip served as a time control. The phenoxybenzamine was then washed out and washes

repeated every 5 minutes for the next ninety minutes. Cumulative dose-responses were then re-obtained in each strip to the same sympathomimetic amine.

VIII. DETERMINATION OF UPTAKE OF ISOPRENALINE AND NORADRENALINE IN CAT SPLEEN

Uptake of isoprenaline and noradrenaline was measured in spleen strips prepared as already described but taken from cats depleted of catecholamine stores by reserpine (1 mg/kg) injected intraperitoneally 24 hours before the experiment or by denervation of the spleen fourteen days previously. Noradrenaline content was assayed by the method of Euler and Lishajko (1961). Isoprenaline was assayed by a similar technique with a standard curve for isoprenaline made daily; the wavelengths used in adrenaline estimations (435 mμ exciting, 540 mμ reading) gave the best results. Recoveries of known amounts of isoprenaline and noradrenaline were similar, between 88.6 and 92.2%. With these data tissue levels of isoprenaline were determined. If present adrenaline or noradrenaline interfered with the estimation of isoprenaline, but in the treated spleens the adrenaline and noradrenaline contents were lower than the amounts detectable by the method used (2×10^{-9} g/g tissue) and so did not interfere with the estimations of isoprenaline.

IX. DETERMINATION OF POTASSIUM, SODIUM AND WATER CONTENT

A lidded platinum crucible was weighed without and with a tissue strip, placed in an oven at 150°F for twenty four hours, removed and allowed to cool in a dessicator and reweighed. This procedure was repeated till a constant weight obtained. The crucibles with lid on were then placed in an incinerator at 200°C for 2 hours, then the temperature increased to 400°C for 2 hours and finally the temperature

increased to 600°C for twenty hours. The gradual increase in temperature reduces spluttering of tissue. The crucibles were placed in a dessicator to cool then the residue dissolved in 0.75 ml of 12 N hydrochloric acid. The resulting solution was then appropriately diluted with glass distilled water and the sodium and potassium concentrations were determined by a flame photometer.

X. STATISTICAL ANALYSIS

Statistical significance was determined by the t test for paired observations (Goldstein, 1964) in experiments where tests were done with a strip used as its own control. In comparison between different strips from the same or different cats statistical significance was determined by Student's t test. All means are given with their standard errors. P values were obtained from a two tailed t table (Steel and Torrie, 1960).

Comparisons between treatments of full dose-response curves were analysed from the geometric means of ED_{50} by analysis of variance and Duncan's multiple range tests (Steel and Torrie, 1960).

D. DRUGS AND SOLUTIONS

The compounds used in this study and the sources from where they were obtained are listed below. All solutions were made weight/volume in terms of the base unless otherwise specified. Stock solutions were stored at 4°C. Concentrations mentioned in the text are final concentrations in the bath fluid in g/ml. At no time was more than 1 ml of a testing solution added to the tissue bath.

Agonists

Stock solutions were made in 0.01 M HCl. On the morning of

use, the stock solutions were diluted as required with acidified 0.9% sodium chloride solution.

TABLE 1

Acetylcholine chloride	Calbiochem
l-Adrenaline bitartrate	Mann Research Laboratories
Histamine diphosphate	Nutritional Biochemical Corporation
5-Hydroxytryptamine creatinine sulphate	Calbiochem
d-Isoprenaline bitartrate	Winthrop Laboratories
dl-Isoprenaline bitartrate	Winthrop Laboratories
l-Isoprenaline bitartrate	Winthrop Laboratories
Methoxamine hydrochloride	Burroughs Wellcome & Co., Inc.
l-Noradrenaline bitartrate monohydrate	Calbiochem
dl α-methyl noradrenaline hydrochloride	Winthrop Laboratories
l-Phenylephrine hydrochloride	Winthrop Laboratories
Tyramine hydrochloride	Calbiochem

Antagonists

Stock solutions of the salt were made in distilled water of cocaine hydrochloride, British Drug Houses, diphenhydramine hydrochloride, Parke Davis & Co., and phentolamine hydrochloride, Aldrich Chemical Company, Inc. Suitable dilutions were made daily in 0.9% sodium chloride solution.

Other Drugs

Angiotensin amide (Hypertensin Ciba) was prepared to the appropriate concentration from vials containing angiotensin amide 2.5 mg, mannitol 47.4 mg and thimerosal 0.1 mg and the solution stored at 4°C.

Phenoxybenzamine hydrochloride (POB) (Smith, Kline & French) was kept as a stock solution containing 1 mg/ml in propylene glycol and 0.1 M HCl. Dilutions when required were made in acidified 0.9% NaCl solution.

A stock solution of reserpine (Ciba) containing 5 mg of the base/ml was prepared for intraperitoneal injection; 100 mg reserpine was dissolved 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.

For oxytocin (Syntocinon, Sandoz), appropriate dilutions were made in 0.9% NaCl on the day of use from ampoules containing 10 IU/ml of synthetic oxytocin (1.0 IU approximately equals 20 μg oxytocin).

Bathing Media

The bathing fluid for the spleen and ileum was Krebs-Henseleit solution of the following composition:

TABLE 2

SUBSTANCE	CONCENTRATION	
	g/l	mM
NaCl	6.9	118
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

The solution was saturated with a mixture of 95% O_2 and 5% CO_2 before the experiment and this mixture was also bubbled through the fluid in each organ bath and the main reservoir during the experiment.

The bathing fluid for the rat uterus was a modified Dale's solution of the following composition.

TABLE 3

SUBSTANCE	CONCENTRATION	
	g/l	mM
NaCl	9.0	153.9
KCl	0.42	5.6
$CaCl_2$	0.06	0.54
$NaHCO_3$	0.50	5.7
Glucose	0.50	2.75

The solution was saturated with a mixture of 95% O_2 and 5% CO_2 before the experiment and this mixture was also bubbled through the fluid in each organ bath and the main reservoir during the experiment.

SECTION III

RESULTS

RESULTS

Preliminary experiments were done to determine the sensitivity of cat spleen to various biogenic amines and the duration of viability of spleen strips *in vitro*.

VARIABILITY OF RESPONSES OF CAT SPLEEN STRIPS

In preliminary experiments response curves to a stimulating drug in several strips from a single spleen in some cases showed little variation between strips, in other experiments they varied markedly. Strips were carefully cut to the same length and width and variation was not therefore due to differences in these dimensions. Another possible variable was thickness of strips and examination showed that in cats greater than 2.0 kg spleens varied in thickness around the edge of the spleen, where strips were cut. Strips from cats less than 0.8 kg were generally less sensitive to stimulating agents.

We compared between two age groups designated as adults (>2.0 kg) and kittens (0.8 - 1.5 kg). Four strips of the same length and width were cut from a single spleen, weighed and a statistical analysis done between 24 animals in each age group (Table 4).

The mean weights of strips in the two groups were significantly different ($P<0.005$). Analysis of variance also showed a significant difference ($P<0.001$) between the two groups.

DURATION OF VIABILITY OF THE SPLEEN STRIP

Several of the proposed experiments required repeated full dose-response curves to a single stimulating agent or to different drugs and accordingly experiments often lasted several hours. The

TABLE 4

COMPARISON OF WEIGHTS OF CAT SPLEEN STRIPS BETWEEN KITTENS (<1.5 kg) AND ADULTS (>2.0 kg)

Each Value Represents Mean Weight of Strips From One Spleen

	KITTENS (24 animals)	ADULTS (24 animals)
	0.1517	0.1492
	0.1493	0.2447
	0.1552	0.1864
	0.1475	0.3217
	0.1562	0.1984
	0.1491	0.1957
	0.1399	0.3306
	0.1522	0.1864
	0.1507	0.1563
	0.1486	0.1746
	0.1493	0.2518
	0.1397	0.3007
	0.1482	0.2816
	0.1479	0.2942
	0.1503	0.1666
	0.1496	0.3216
	0.1500	0.2715
	0.1493	0.2536
	0.1490	0.1982
	0.1584	0.2214
	0.1577	0.3017
	0.1497	0.2643
	0.1538	0.1980
	0.1395	0.1885
Mean ± S.E.	0.1497 ± 0.001	0.2357 ± 0.012

viability of the preparation was therefore tested over a period of several hours.

The criterion chosen for viability of the spleen strip was maintenance of normal ionic content tested by analysis of sodium and potassium content. Comparisons were made between two age groups (>2.0 kg) and (0.8 - 1.5 kg).

Viability of spleen strips from both age groups was tested as follows. Three strips were cut from one spleen and analysed for sodium and potassium content; one strip was analysed immediately, the other two placed in organ baths. One strip had the bathing fluid changed every five minutes, the other was also stimulated every twenty minutes by addition of small doses of a stimulating drug, e.g. noradrenaline 10^{-6} g/ml. Results of six experiments are shown in Tables 5 and 6. The normal sodium and potassium contents, i.e. of the strips analyzed immediately, did not differ significantly between the two age groups (Table 5). Results of the experiments which determined that no significant changes in sodium or potassium content occurred in less than the reported times are not shown.

Strips from adult cats placed in an organ bath and the fluid changed every five minutes showed a significant ($P<0.01$) gain of sodium and loss of potassium after three hours. Strips which were additionally stimulated by a drug took more than five hours for the same changes to occur. The times required for similar changes in sodium and potassium in strips from kittens were greater than five and eight hours respectively.

The standard procedure of preparing spleen strips involved

TABLE 5

POTASSIUM AND SODIUM CONTENT OF CAT SPLEEN

COMPARISON BETWEEN ADULT (>2.0 kg) AND KITTEN (<1.5 kg)

	Kitten		Adult	
	Sodium mEq/kg	Potassium mEq/kg	Sodium mEq/kg	Potassium mEq/kg
	123.24	82.77	123.25	98.47
	111.17	100.66	119.92	94.53
	118.47	101.74	124.17	116.11
	127.24	112.33	106.33	103.47
	119.63	98.37	121.46	85.91
	117.45	93.56	123.10	89.18
Mean ± S.E.	119.53 ± 2.22	98.24 ± 3.99	119.70 ± 2.74	97.94. ± 4.45

TABLE 6

POTASSIUM AND SODIUM CONTENT OF CAT SPLEEN

EFFECT OF LEAVING SPLEEN STRIP IN ORGAN BATH AT 37°C WITH AND WITHOUT STIMULATION BY AN AGONIST

	UNTREATED		WITHOUT STIMULATION		WITH STIMULATION	
	SODIUM	POTASSIUM	SODIUM	POTASSIUM	SODIUM	POTASSIUM
Adult (>2.0 kg)	119.71 ± 2.74	97.94 ± 4.45	>3 hr 133.01 ± 4.94	74.04 ± 4.77	>5 hr 136.21 ± 3.99	70.79 ± 4.93
Kitten (<1.5 kg)	119.53 ± 2.22	98.24 ± 3.99	>5 hr 138.17 ± 5.21	74.92 ± 4.91	>8 hr 131.37 ± 4.09	71.41 ± 4.83

dissection in Krebs-Henseleit solution at 4°C. Exposure to such low temperatures is known to inhibit metabolic processes required to maintain normal ionic states in excitable tissues. Recovery of normal ionic states was tested in the following experiments.

Three strips were cut from the same spleen and analysed for sodium and potassium content after the following procedures. Strip one, immediately on removal from the animal; strip two, after fifteen minutes in Krebs-Henseleit solution at 4°C; and strip three, after exposure at 4°C placed in an organ bath containing Krebs-Henseleit solution at 37°C. Results of six such experiments are shown in Table 7.

Strips after exposure to a temperature of 4°C show a significant ($P<0.01$) gain of sodium and loss of potassium compared to untreated strips. After twenty minutes at 37°C such strips have lost sodium and regained potassium so that there is no significant difference from untreated strips.

The use of animals in the weight range 0.8 to 1.5 kg, with strips cut the same length and width, reduced variation between strips from the same or different spleens to less than five per cent and ensured a viable preparation for at least eight hours as indicated by sodium and potassium content. All further experiments unless otherwise stated were done on cats weighing 0.8 to 1.5 kg.

RESPONSE OF SPLEEN STRIP TO AGONISTS

The sensitivity of cat spleen to various stimulating agents was tested in the following manner. Full dose-response curves to acetylcholine, angiotensin, histamine and noradrenaline were done in four strips from each of six spleens. Each strip from one spleen was exposed to only one drug to avoid problems of desensitization. Typical dose-response

TABLE 7

POTASSIUM AND SODIUM CONTENT OF CAT SPLEEN

EFFECT OF PLACING IN KREBS-HENSELEIT SOLUTION AT 4°C AND THEN IN KREBS-HENSELEIT SOLUTION AT 37°C

	Untreated		15 min. at 4°C		20 min. at 37°C	
	Sodium	Potassium	Sodium	Potassium	Sodium	Potassium
	123.24	82.77	147.21	55.91	101.50	84.00
	111.17	100.66	138.42	68.47	116.80	116.22
	118.47	101.74	136.55	84.16	110.00	95.29
	127.24	112.33	151.24	70.73	124.21	104.31
	119.63	98.37	141.63	80.19	114.73	97.62
	117.45	93.56	139.71	75.51	116.92	94.05
Mean ± S.E.	119.56 ± 2.22	98.24 ± 3.99	142.46 ± 2.30	72.49 ± 4.08	112.86 ± 3.22	98.58 ± 4.43

curves to the agonists are shown in Fig. 1.

MAXIMUM RESPONSES TO AGONISTS

The concept of spare receptors (Nickerson, 1956; Stephenson, 1956) is generally accepted. If spleen had spare receptors what were the limiting factor(s) that prevented continued response of spleen till 100% receptor occupancy was obtained? One possibility was, maximum responses to agonists were limited by contractile capability of spleen strips. This was tested by the following procedures.

Full dose-response curves to various agonists were obtained in spleen strips and when maximum responses were reached, a second class of agonist was added; e.g. dose-response curve to noradrenaline and histamine added as the second agent. Results of these experiments (Fig. 2) clearly show spleen strips capable of further contractile response than that produced by the maximal dose of one single type of agonist.

An interesting phenomenon was noted that when bathing fluid was changed to remove stimulating drugs, tissues gave a further contraction. Results of experiments where bathing fluid was changed after maximum responses were obtained to agonists are shown in Fig. 3. Similar results were obtained in strips made supersensitive by cocaine, reserpine and denervation (Fig. 4).

Response to the change of bathing fluid was not seen unless an agonist was present in a concentration greater than ED80. pH and temperature of bathing fluid did not change during wash out; stretch was not responsible because wash out responses still occurred when stretch was prevented by an overflow washout procedure.

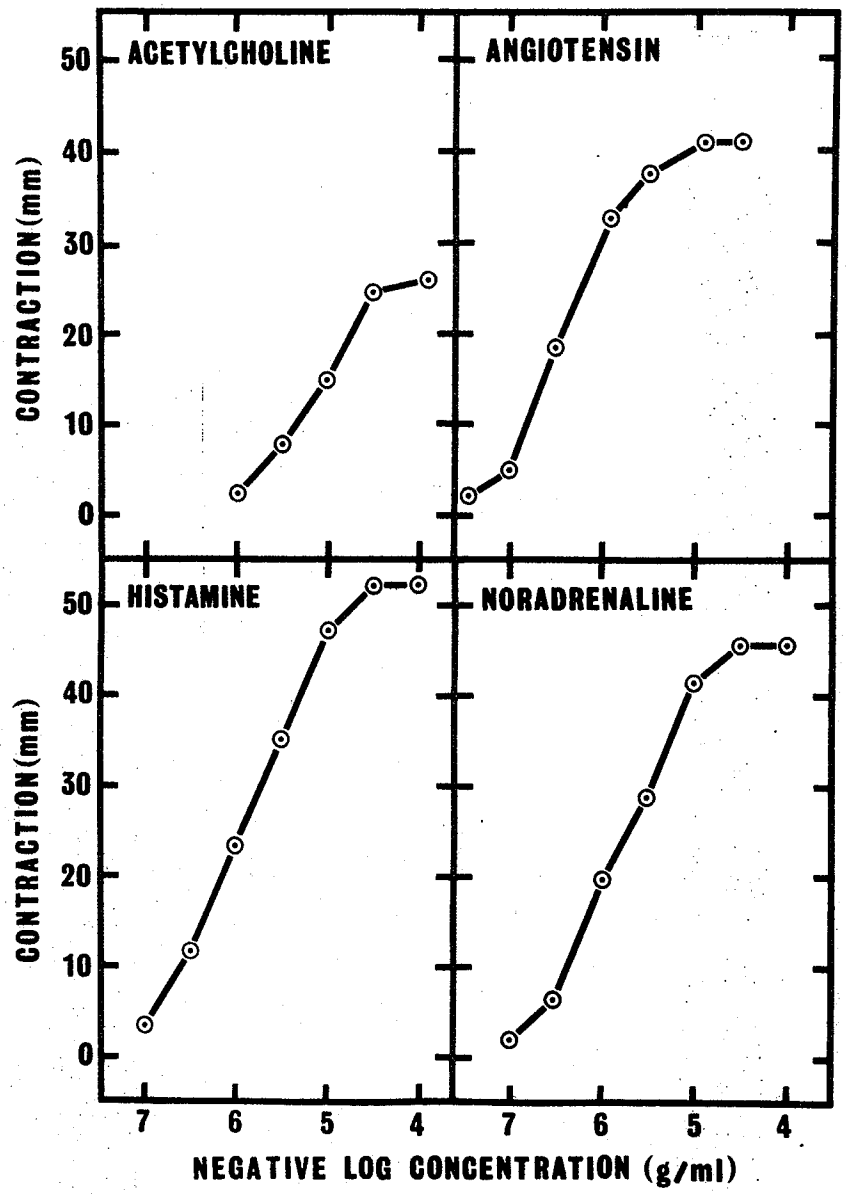


Fig. 1. Dose-response curves to acetylcholine, angiotensin, histamine and noradrenaline in cat spleen.

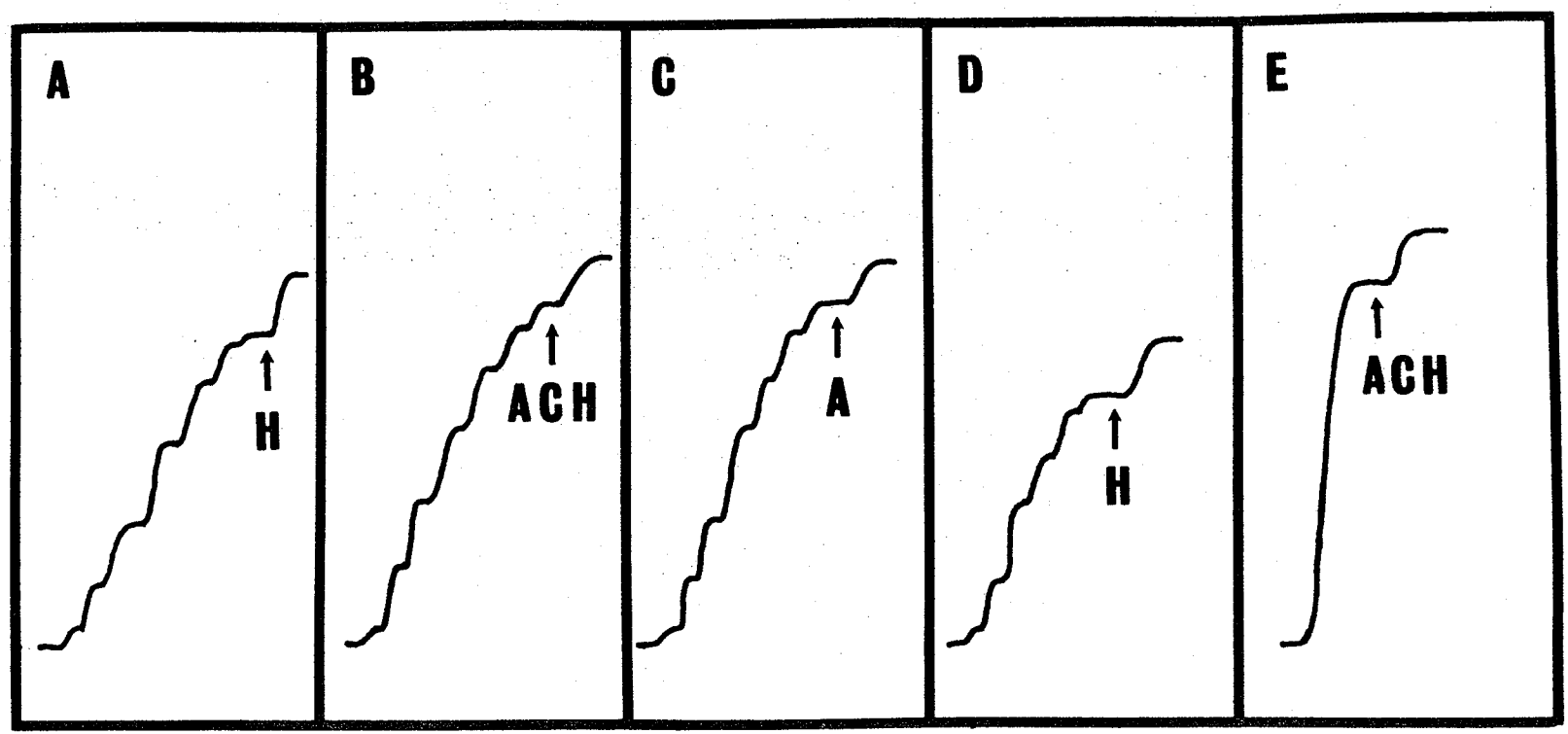


Fig. 2. Effect of addition of one class of agonist on maximum response to another class of agonist in cat spleen.

A. Dose-response curve to adrenaline; Histamine 5 x 10^{-5} g/ml was added at↑

B. Dose-response curve to adrenaline; Acetylcholine 3 x 10^{-5} g/ml was added at↑

C. Dose-response curve to histamine; Adrenaline 3 x 10^{-5} g/ml was added at↑

D. Dose-response curve to acetylcholine; Histamine 3 x 10^{-5} g/ml was added at↑

E. Response to maximal dose of Potassium; Acetylcholine 3 x 10^{-5} g/ml was added at↑

SPARE RECEPTORS IN CAT SPLEEN

All tissues do not have spare receptors. Offenheimer and Ariens (1966) reported rat stomach strip has no spare 5-HT receptors; Lewis and Miller (1966) showed rat seminal vesicle has no spare adrenergic receptors.

The following experiments were done to determine if cat spleen had spare adrenergic receptors.

Cumulative dose-response curves were obtained for one sympathomimetic amine on four spleen strips from one cat. Three strips were exposed to phenoxybenzamine, 10^{-10}, 10^{-9} and 10^{-8} g/ml respectively, for 5 minutes and all tissues then washed every 5 minutes for 1 hour. Dose-response curves were then repeated in all tissues, the tissue not exposed to phenoxybenzamine served as a time control.

Fig. 5 shows result of a typical experiment, the sympathomimetic amine tested was adrenaline. Similar results were obtained with isoprenaline, noradrenaline, nordefrine, phenylephrine and tyramine.

Results of 5 experiments with each sympathomimetic amine show that maximum responses were reduced by phenoxybenzamine even where the threshold concentration of agonist was unchanged. This indicates little or no adrenergic receptor reserve in cat spleen.

SYMPATHOMIMETIC AMINES: FULL AND PARTIAL AGONISTS

Dose-response curves to seven sympathomimetic amines are shown in Fig. 6. These sympathomimetic amines which produced the same maximum response, adrenaline, isoprenaline, noradrenaline and nordefrine are full agonists with an intrinsic activity, α equals 1 (Ariens, 1954). Methoxamine, phenylephrine and tyramine gave lower maximum responses and are

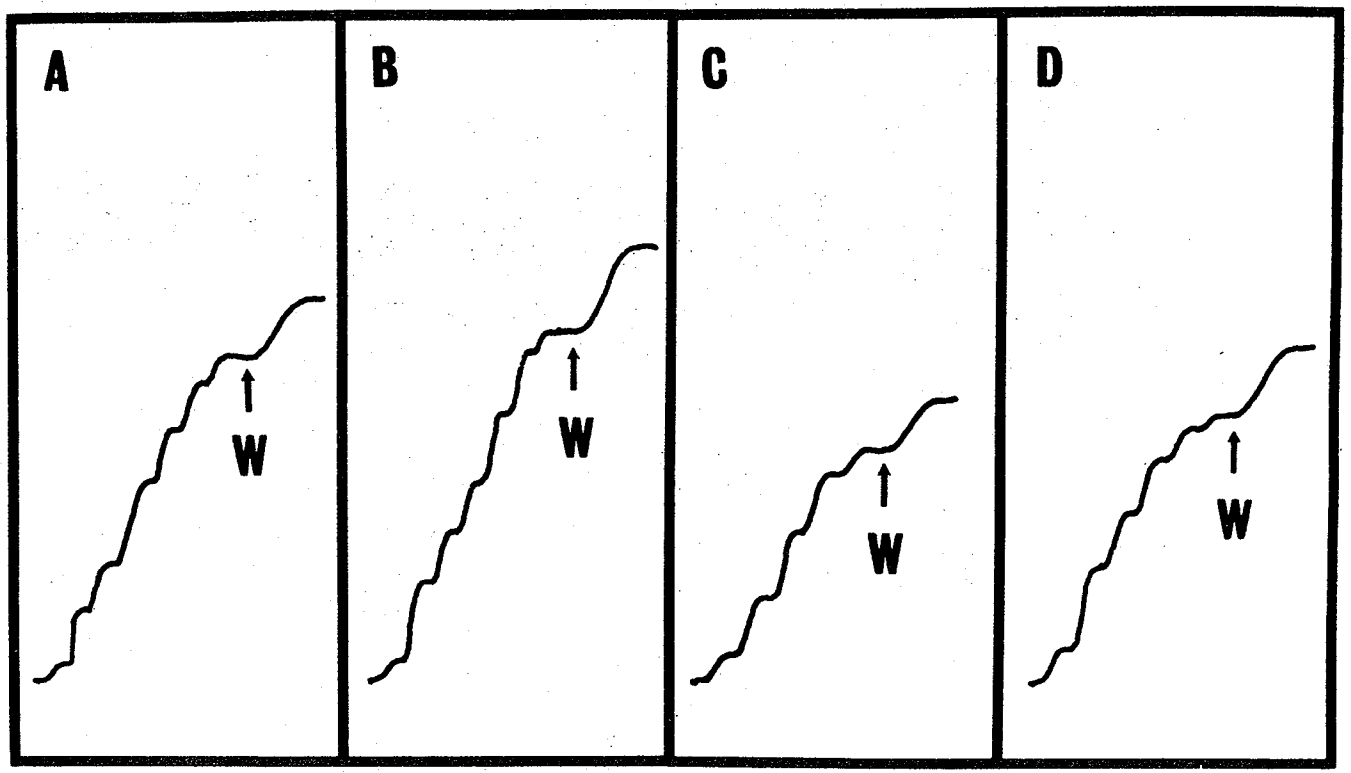


Fig. 3. Effect of changing bathing fluid on maximum response to agonists on cat spleen.

A. Noradrenaline;
B. Histamine;
C. Acetylcholine;
D. Angiotensin;
Bathing fluid was changed at ↑

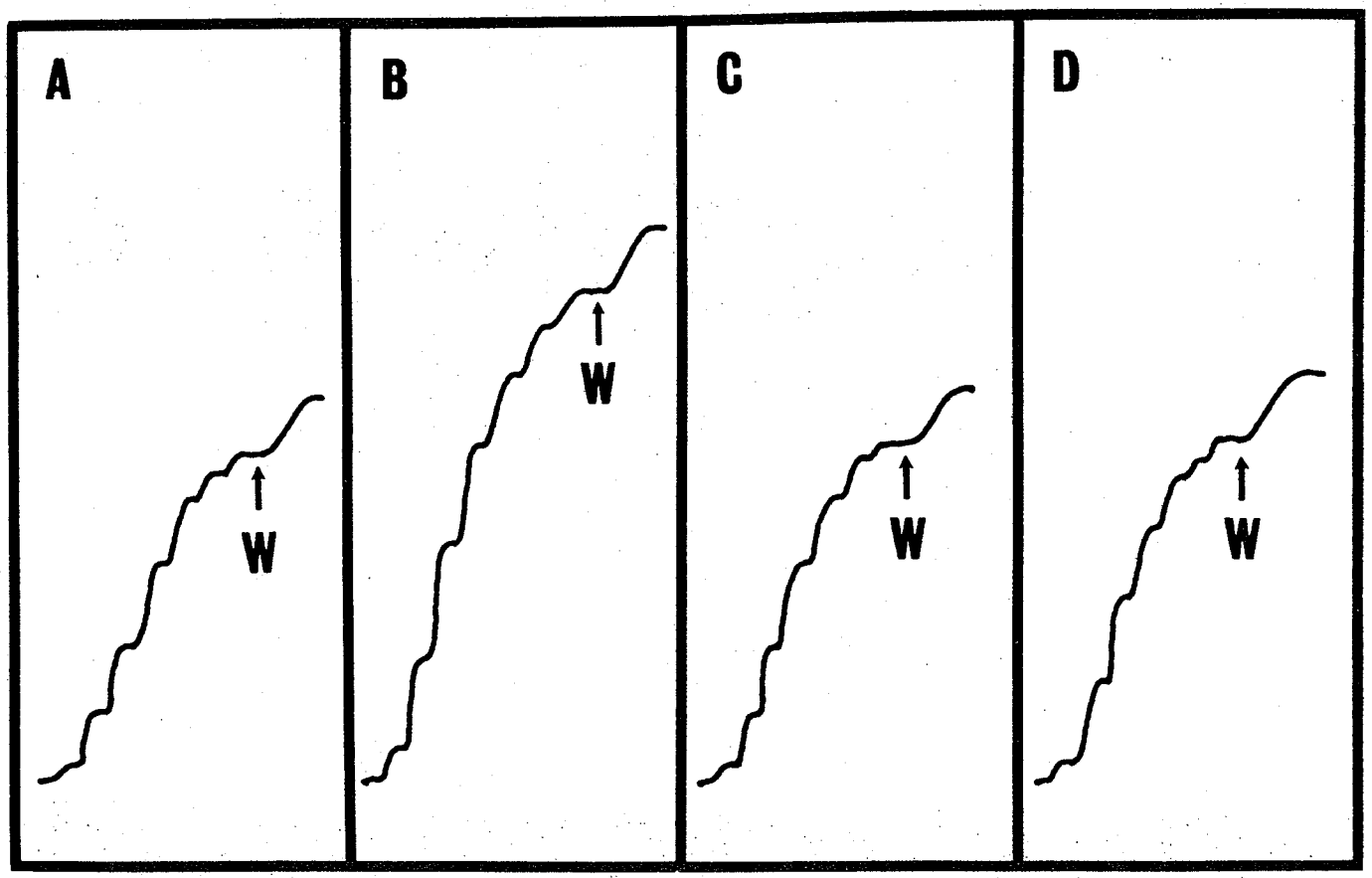


Fig. 4. Effect of changing the bathing fluid on maximum response to noradrenaline in cat spleen.

A. Normal untreated spleen.
B. Spleen from animal treated with reserpine 1 mg/kg, 24 hr previously.
C. Spleen exposed in vitro to cocaine 3×10^{-5} g/ml.
D. Spleen denervated 14 days previously.
The bathing fluid was changed at ↑.

partial agonists, intrinsic activity, α less than 1.

This only holds true if amines tested all act on same receptors, i.e. alpha adrenergic receptors. If different agonists give the same pA_x value for the same antagonist in a given tissue, it is strong evidence that they act on a common receptor, (Arunlakshana and Schild, 1959; Van Rossum and Ariens, 1959a; Jenkinson, 1960). Accordingly pA_3 values against phentolamine were determined with histamine and acetylcholine, which act on other discrete types of receptor in cat spleen (Innes, 1962), to check specificity of antagonism.

Results of these experiments are shown in Table 8. Figures given are mean values, total number of determinations and their standard deviation being indicated in the table. Only one determination was done with methoxamine because of desensitization problems. Repeated tests at any dose level always gave diminished responses.

pA_3 values of phentolamine against the sympathomimetic amines were very similar and, on this basis, can be regarded as acting as a common receptor. Phentolamine sharply discriminates this group from other agonists, acetylcholine and histamine.

AFFINITY OF SYMPATHOMIMETIC AMINES FOR ALPHA ADRENERGIC RECEPTOR

The finding that cat spleen has little or no adrenergic receptor reserve allowed comparisons of relative affinities of the sympathomimetic amines producing the family of dose-response curves shown in Fig. 6. Because in a tissue with no spare receptors, the position of the response-curve of a full agonist should be determined by the affinity of the agonist, whereas changes in affinity and intrinsic activity could determine the position of curves to partial agonists. The ability of each

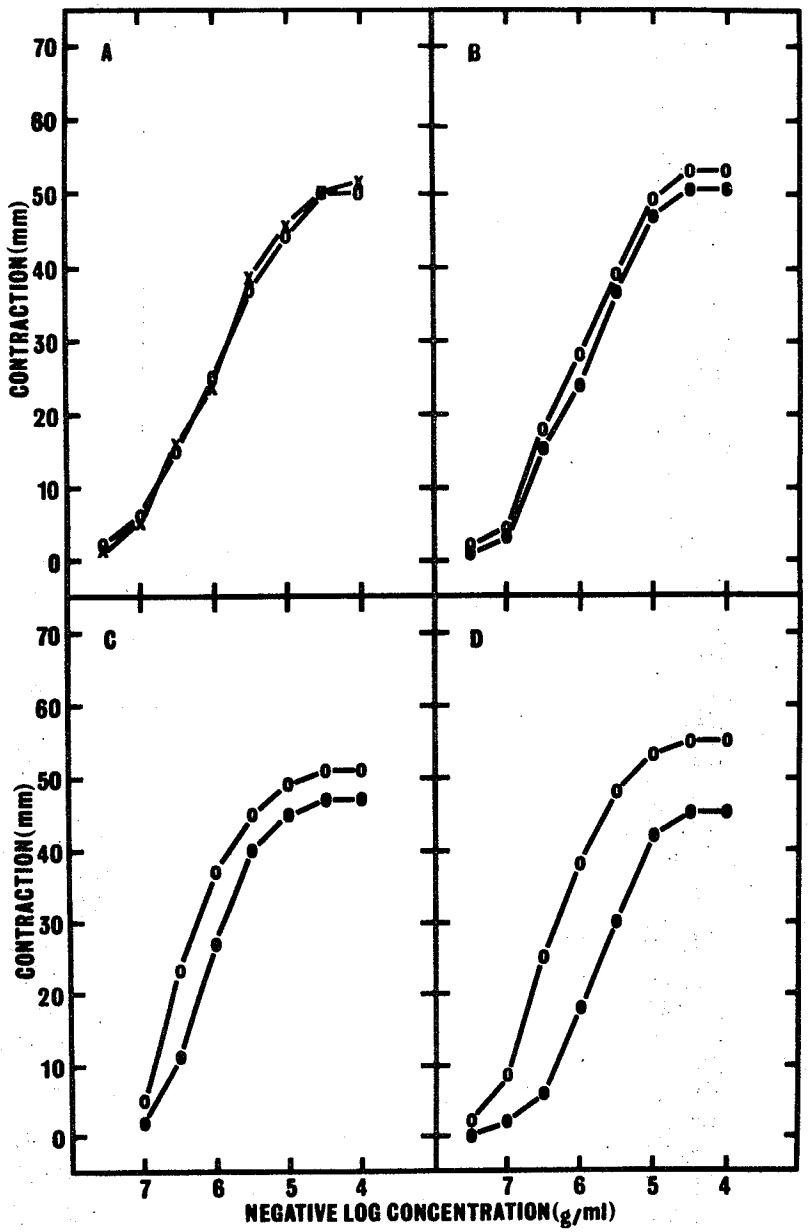


Fig. 5. Effect of phenoxybenzamine on dose-response curves to adrenaline in four strips from the same cat spleen.

A. 0 - 0, Control; X - X Effect of time.

B, C, and D. 0 - 0, Before Phenoxybenzamine; ● - ●, After Phenoxybenzamine 10^{-10}, 10^{-9}, 10^{-8} g/ml respectively.

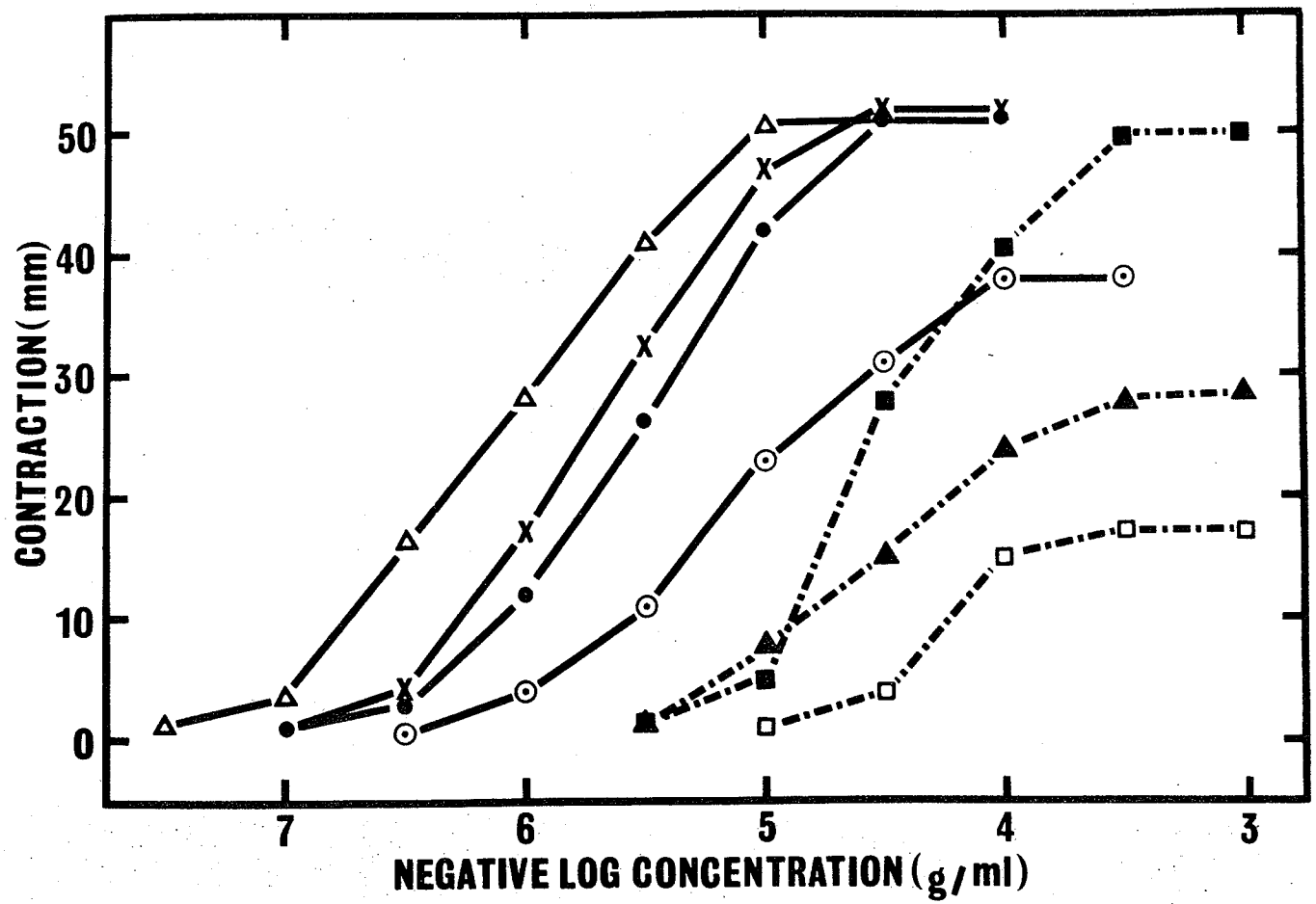


Fig. 6. Dose-response curves to sympathomimetic amines in cat spleen.

△—△ Adrenaline;
X—X Noradrenaline;
●—● Nordefrine;
○—○ Phenylephrine;
■- - -■ Isoprenaline;
▲- - -▲ Tyramine;
□- - -□ Methoxamine.

sympathomimetic amine to protect against phenoxybenzamine was used to estimate its affinity (see Method Section, p.21, for full details of experimental design). Comparisons were made at maximum response before and after phenoxybenzamine; in each case there will be 100 per cent receptor occupancy.

Results of these experiments are shown in Table 9. Adrenaline afforded greater protection ($P<0.0025$) than any other agonist tested. There was no significant difference in protection provided by noradrenaline and nordefrine but each gave greater protection ($P<0.05$) than phenylephrine, tyramine and isoprenaline. Similarly phenylephrine and tyramine showed no significant difference in the protection they provided but gave greater protection ($P<0.05$) than isoprenaline which gave no significant protection compared to unprotected strips.

POTENTIATION OF RESPONSES TO AGONISTS BY COCAINE IN CAT SPLEEN

Cocaine potentiates responses to catecholamines in many effector organs in most species, but there is not yet agreement on the ability of cocaine to potentiate responses to other classes of agonists (Rosenblueth, 1932; Tsai *et al.*, 1968). Accordingly we tested the ability of cocaine to potentiate responses to acetylcholine, histamine and noradrenaline, agonists known to act on different types of receptors in cat spleen (Innes, 1962).

Full dose-response curves for acetylcholine, histamine and noradrenaline were obtained before and with cocaine 10^{-6} and 3×10^{-5} g/ml. Four strips were cut from each of twelve spleens. Only two agonists were tested on any one spleen. Two strips were tested with noradrenaline, two with either acetylcholine or histamine. For each of the two agonists one

TABLE 8

ANTAGONISM BY PHENTOLAMINE IN CAT SPLEEN

Agonist	pA_3 Values (5 min Exposure time) (Mean ± S.E.)	No. of Determinations
Adrenaline	6.95 ± 0.03	24
Noradrenaline	6.92 ± 0.02	18
Nordefrine	6.97 ± 0.03	9
Phenylephrine	6.91 ± 0.04	11
Tyramine	6.96 ± 0.03	8
Isoprenaline	6.97 ± 0.04	14
Methoxamine	6.93	1
Histamine	4.51 ± 0.12	13
Acetylcholine	3.85 ± 0.21	9

TABLE 9

AFFINITY OF SYMPATHOMIMETIC AMINES FOR THE ALPHA ADRENERGIC RECEPTOR OF CAT SPLEEN ESTIMATED BY PROTECTION AFFORDED AGAINST PHENOXYBENZAMINE BLOCKADE

Protecting Agent 1×10^{-4} g/ml	Protection Expressed as a Percentage of Maximum Response Before and After Phenoxybenzamine (2.5×10^{-7} g/ml)
Adrenaline (19)	71.13 ± 3.85
Noradrenaline (19)	58.83 ± 2.67
Nordefrine (13)	56.66 ± 3.66
Tyramine (7)	45.40 ± 2.17
Phenylephrine (7)	42.46 ± 2.34
Isoprenaline (9)	32.60 ± 4.1
Unprotected (19)	29.01 ± 3.43

( ) indicates number of experiments

strip was retested with cocaine, the other served as time control.

Acetylcholine and histamine often gave desensitization problems, in that the repeated dose-response curve in the time control was shifted to the right, and the results presented are from experiments where time controls did not show desensitization. The time required between dose-response curves to do this successfully was between six to eight hours, close to the time limit of viability of spleen strip (see p.28). Angiotensin was not tested with full dose-response curves because even with 10 hours between testing dose-response curves, desensitization was still present. However cocaine could be added when the response to a single test concentration of angiotensin had plateaued and responses to angiotensin were potentiated ($P<0.05$) by cocaine 3×10^{-5} g/ml.

Cocaine 10^{-8} to 10^{-6} g/ml potentiated only noradrenaline responses; cocaine 3×10^{-5} or 10^{-4} g/ml potentiated responses to acetylcholine, histamine and noradrenaline. Maximum potentiation was obtained at 3×10^{-5} g/ml cocaine. Results and statistical analysis of twelve experiments with acetylcholine, histamine and noradrenaline are shown in Fig. 7 and Table 10 where the concentrations of cocaine used were 10^{-6} or 3×10^{-5} g/ml. Potentiation of acetylcholine and histamine was significantly less ($P<0.0025$) than that of noradrenaline. Maximum responses to agonists tested were unchanged by cocaine.

EFFECT OF COCAINE ON ANTAGONISM BY PHENTOLAMINE

Clark (1937) and other workers since then have suggested that cocaine might cause potentiation by changing the affinity of the alpha adrenergic receptor so that the affinity for agonists was increased. We have shown that cocaine 10^{-6} g/ml specifically potentiates responses to

catecholamines, whereas cocaine 3×10^{-5} g/ml gives an unspecific potentiation. Accordingly we determined pA_3 values against phentolamine with and without cocaine, using adrenaline, a full agonist, and phenylephrine, a partial agonist, agents acting on alpha adrenergic receptors, and histamine an agent acting on another type of receptor (Innes, 1962).

pA_3 values before and in the presence of cocaine 10^{-6} or 3×10^{-5} g/ml were compared in thirty experiments. When adrenaline or phenylephrine was the agonist it was possible to determine pA_3 value without cocaine and then wash the tissue till control responses had returned and then repeat the determination in the presence of cocaine. This was not possible with histamine because of desensitization. Accordingly, pA_3 values with histamine were determined on separate strips with or without cocaine.

Results of these experiments are shown in Table 11. There was no significant change in pA_3 values from controls in the presence of cocaine 10^{-6} or 3×10^{-5} g/ml.

EFFECT OF RESERPINE PRETREATMENT

Effects of reserpine on responses to agonists could not be determined within a single strip; therefore responses of strips from cats given reserpine (1 mg/kg 24 hr before the experiment) were compared with responses of strips from untreated cats.

Full dose-response curves were obtained to acetylcholine, angiotensin, histamine and noradrenaline in spleen strips from treated and untreated animals. Only one agonist was tested per strip. Fig. 8 and Table 12 show results and statistical analysis of fourteen experiments.

Responses to all agonists tested were significantly potentiated.

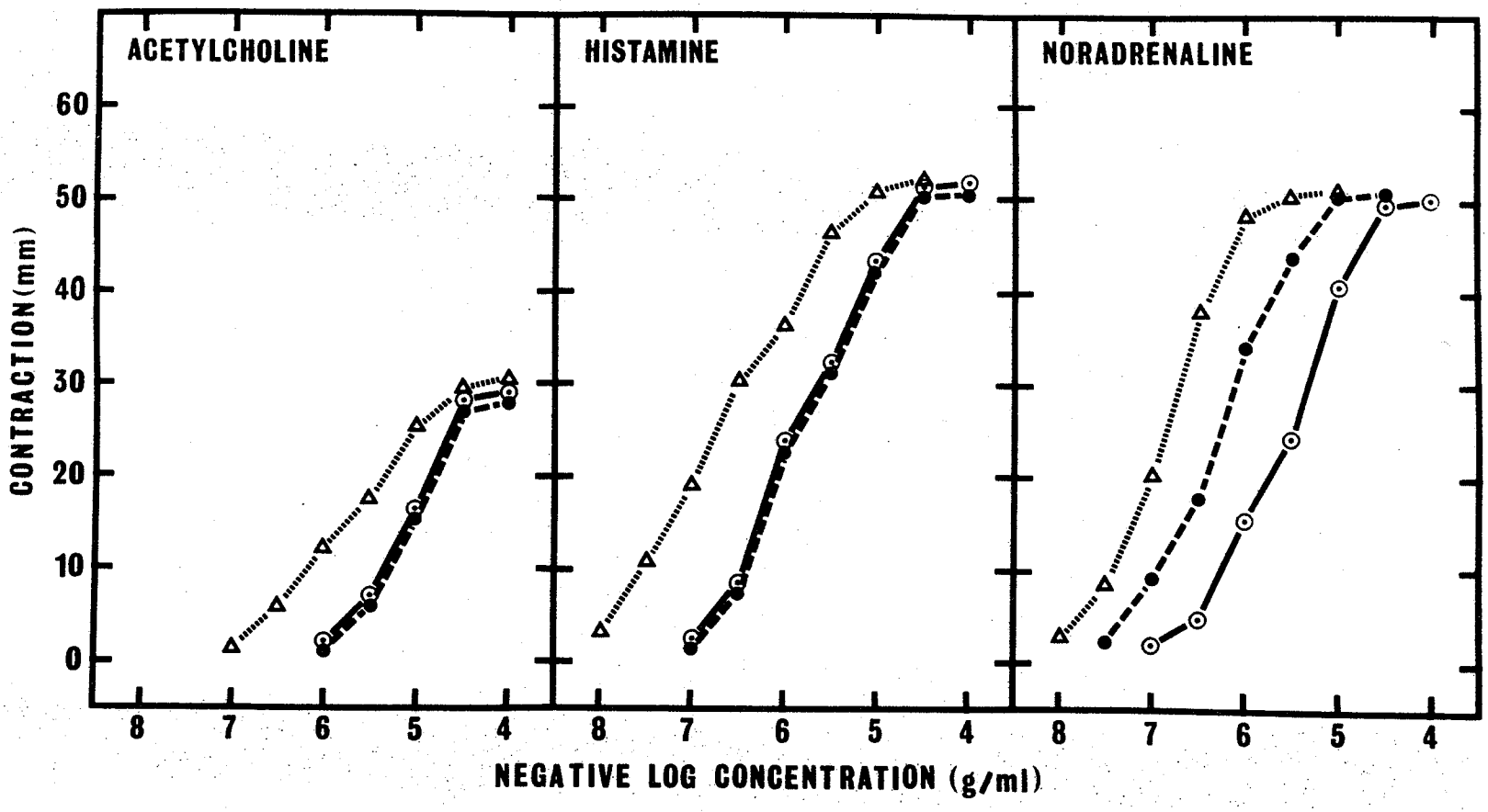


Fig. 7. Effect of cocaine on dose-response curves to acetylcholine, histamine and noradrenaline in cat spleen.
Responses from normal control spleen, O—O, are compared with responses with cocaine 10^{-6} g/ml, ●– –●, and with cocaine 3×10^{-5} g/ml, △····△.

TABLE 10

POTENTIATION OF ACETYLCHOLINE, HISTAMINE AND NORADRENALINE BY COCAINE (10^{-6} g/ml) AND (3×10^{-5} g/ml)

Drug	Treatment	Mean ED_{50} μg/ml	95% Confidence Limits Lower	95% Confidence Limits Upper	Significance
Acetylcholine	Control	9.70	10.20	8.65	N.S.
	Cocaine 10^{-6} g/ml	9.70	10.17	8.62	
Acetylcholine	Control	9.70	10.20	8.65	P<0.005
	Cocaine 3×10^{-5} g/ml	2.20	2.41	1.82	
Histamine	Control	1.60	1.74	1.43	N.S.
	Cocaine 10^{-6} g/ml	1.60	1.65	1.39	
Histamine	Control	1.60	1.74	1.43	P<0.001
	Cocaine 3×10^{-5} g/ml	0.23	0.26	0.19	
Noradrenaline	Control	3.2	3.51	2.77	P<0.001
	Cocaine 10^{-6} g/ml	0.84	0.89	0.69	
Noradrenaline	Control	3.2	3.51	2.77	P<0.001
	Cocaine 3×10^{-5} g/ml	0.13	0.18	0.09	
Noradrenaline	Cocaine 10^{-6} g/ml	0.84	0.89	0.69	P<0.001
	Cocaine 3×10^{-5} g/ml	0.13	0.18	0.09	

Treatment with reserpine significantly ($P<0.001$) increased maximum responses to all agonists tested compared to controls (Table 13).

FULL AND PARTIAL AGONISTS

The effect of reserpine was tested on responses to adrenaline, a full agonist with the highest affinity for alpha receptors of all the sympathomimetics tested, isoprenaline, a full agonist with low affinity, and phenylephrine, a partial agonist with intermediate affinity.

Responses to full and partial agonists were significantly potentiated. Maximum responses were also potentiated. Results and statistical analysis are shown in Fig. 9 and Tables 13 and 14.

EFFECT OF COCAINE ON HISTAMINE AND NORADRENALINE RESPONSES IN CAT SPLEEN PRETREATED WITH RESERPINE

Cumulative dose-response curves to histamine and noradrenaline before and in the presence of cocaine (10^{-6} and 3×10^{-5} g/ml) were compared in sixteen experiments on strips from cats given reserpine (1 mg/kg intraperitoneally) 24 hours before the experiment. Four strips from the same spleen were used in each experiment; histamine and noradrenaline were each tested on two strips, one strip without cocaine, the second strip in the presence of cocaine. For comparison two strips from a cat which had not been given reserpine were tested at the same time.

Results and statistical analysis are shown in Fig. 10 and Table 15. Cocaine 10^{-6} g/ml specifically potentiated noradrenaline, whereas cocaine 3×10^{-5} g/ml potentiated histamine and further potentiated noradrenaline. The maximum responses in the reserpine treated strips, which were increased when compared with controls, were not changed by cocaine.

TABLE 11

ANTAGONISM BY PHENTOLAMINE IN CAT SPLEEN

Agonist	Treatment	pA_3 Values (5 min Exposure time) (Mean ± S.E.)	No. of Determinations
Adrenaline	Control	6.93 ± 0.04	12
Adrenaline	Cocaine 10^{-6} g/ml	6.95 ± 0.02	6
Adrenaline	Cocaine 3×10^{-5} g/ml	6.92 ± 0.05	6
Phenylephrine	Control	6.92 ± 0.04	12
Phenylephrine	Cocaine 10^{-6} g/ml	6.91 ± 0.02	6
Phenylephrine	Cocaine 3×10^{-5} g/ml	6.94 ± 0.01	6
Histamine	Control	4.57 ± 0.14	12
Histamine	Cocaine 10^{-6} g/ml	4.50 ± 0.17	6
Histamine	Cocaine 3×10^{-5} g/ml	4.53 ± 0.15	6

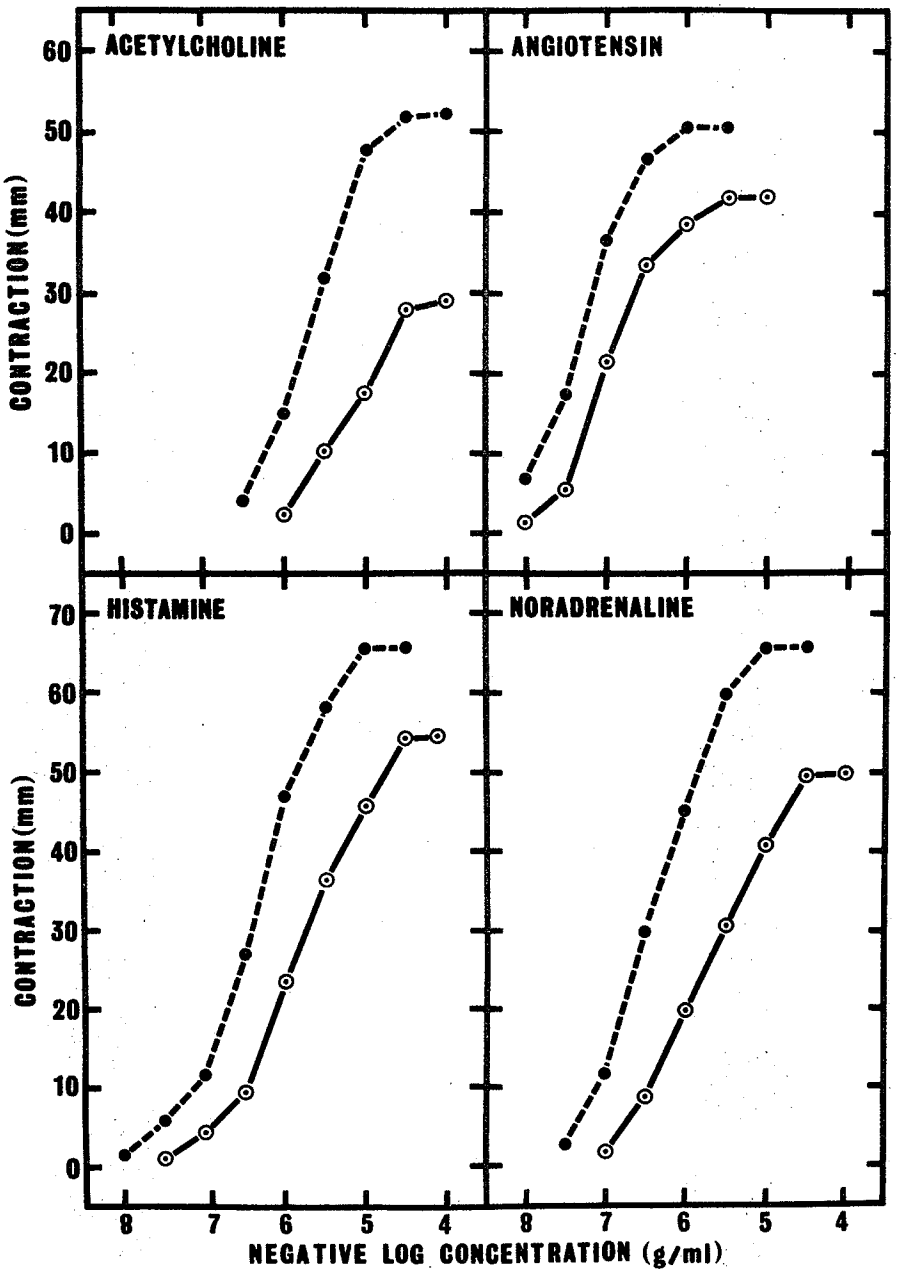


Fig. 8. Effect of reserpine treatment on dose-response curves to acetylcholine, angiotensin, histamine and noradrenaline in cat spleen.
Responses from normal control spleen, O—O, are compared with responses of spleen from cat treated with reserpine (1 mg/kg), ●- -●.

TABLE 12

POTENTIATION OF ACETYLCHOLINE, ANGIOTENSIN, HISTAMINE AND NORADRENALINE BY RESERPINE (1 mg/kg)

Drug	Treatment	Mean ED_{50} $\mu g/ml$	95% Confidence Limits		Significance
			Lower	Upper	
Acetylcholine	Control	8.7	9.2	7.9	P<0.01
	Reserpine	3.2	3.8	2.7	
Angiotensin	Control	1.1	1.27	0.90	P<0.01
	Reserpine	0.58	0.66	0.51	
Histamine	Control	1.7	1.81	1.62	P<0.01
	Reserpine	0.65	0.72	0.59	
Noradrenaline	Control	2.7	2.83	2.64	P<0.001
	Reserpine	0.59	0.64	0.55	

TABLE 13

EFFECT OF RESERPINE ON MAXIMUM RESPONSES TO AGONISTS

Agonist	Treatment	Maximum Responses Mean ± S.E.	Significance
Acetylcholine	Control	29.08 ± 0.90	$P < 0.001$
	Reserpine	52.03 ± 1.36	
Adrenaline	Control	50.03 ± 0.42	$P < 0.005$
	Reserpine	63.50 ± 0.93	
Angiotensin	Control	42.13 ± 0.86	$P < 0.01$
	Reserpine	50.50 ± 0.93	
Histamine	Control	54.50 ± 0.91	$P < 0.005$
	Reserpine	64.50 ± 0.90	
Isoprenaline	Control	49.10 ± 0.46	$P < 0.005$
	Reserpine	63.80 ± 0.82	
Noradrenaline	Control	50.68 ± 0.39	$P < 0.005$
	Reserpine	64.50 ± 1.01	
Phenylephrine	Control	38.60 ± 0.60	$P < 0.005$
	Reserpine	48.35 ± 1.04	

EFFECT OF ANTAGONISTS ON MAXIMUM RESPONSES AFTER RESERPINE

Increased maximum responses to agonists found after treatment with reserpine might have been due to changes in tone of tissues caused by alteration of normal mediator influences; reserpine is known to deplete tissues of their normal catecholamine content (Burn and Rand, 1958b; 1959) and increase acetylcholine content (Fleming et al., 1968). We decided to test the effect of altering mediator influences by the use of antagonists. The antagonists might have produced a potentiating effect on untreated spleen strips, unrelated to changes in tone caused by alteration of mediator influences. Therefore we tested effect of the antagonists in spleen strips from untreated and reserpine treated cats.

Cumulative dose-response curves to histamine and noradrenaline before and in the presence of phentolamine or atropine were compared in sixteen experiments on strips from untreated or reserpine treated animals. Four strips from the same spleen were used in each experiment, histamine and noradrenaline were each tested on two strips, one strip without antagonist, the second strip in the presence of antagonist.

Phentolamine, an alpha adrenergic antagonist, was used when histamine was the agonist, atropine an antimuscarinic agent the antagonist used when noradrenaline was the agonist. The concentration of atropine or phentolamine chosen was approximately the pA_3 values of these agents (against their respective physiological mediators) to provide relatively specific antagonism. Histamine responses were therefore tested with reduced sympathetic influences such as might be expected after reserpine treatment. Noradrenaline was tested with any possible cholinergic influences reduced. Reserpine, in fact, increases or causes no change in

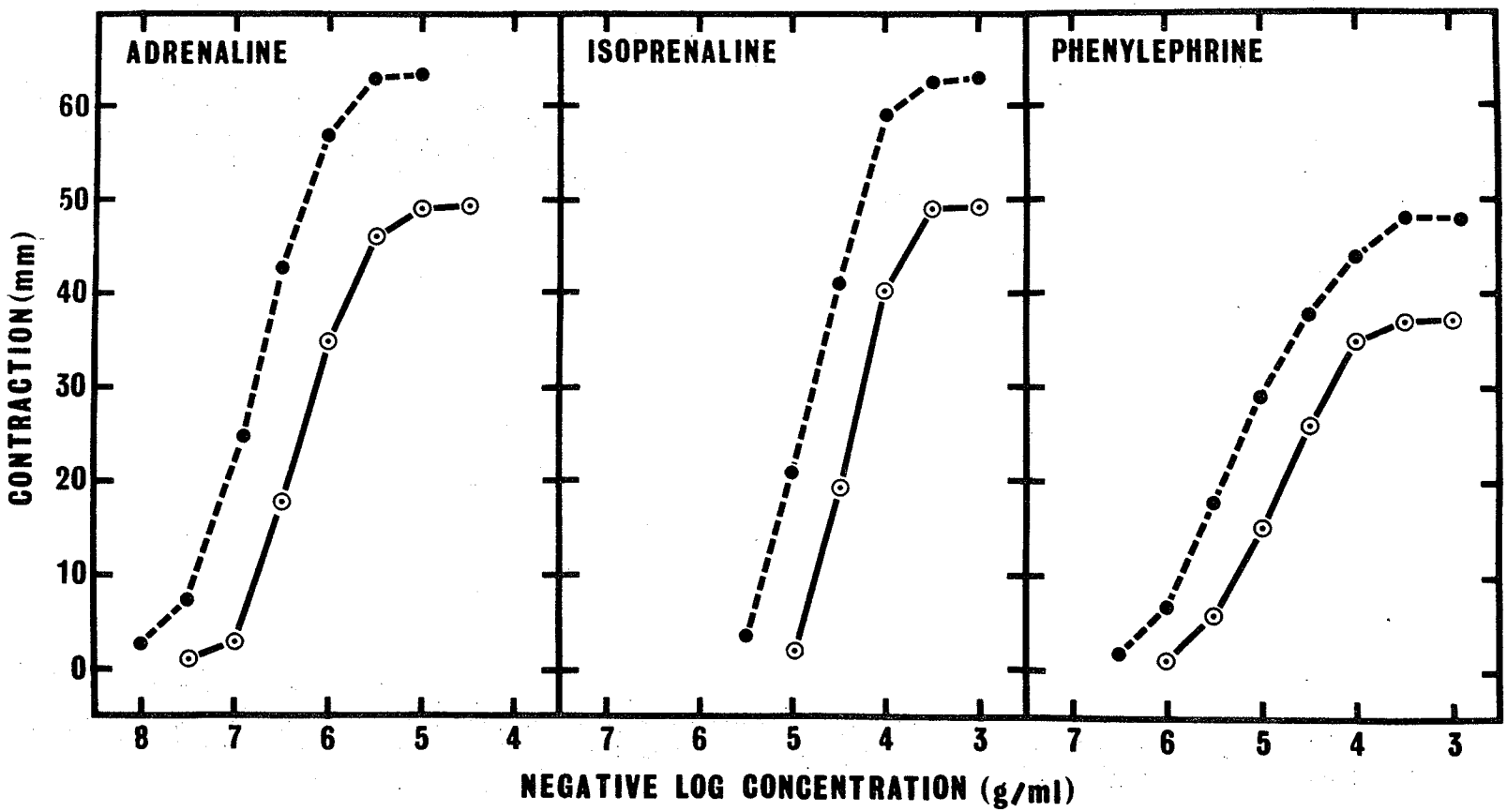


Fig. 9. Effect of reserpine treatment on dose-response curves to sympathomimetic amines with different intrinsic activities in cat spleen.
Responses from normal control spleen, O—O, are compared with responses of spleen from cat treated with reserpine (1 mg/kg), ●- -●.

TABLE 14

POTENTIATION OF ADRENALINE, ISOPRENALINE AND PHENYLEPHRINE BY RESERPINE (1 mg/kg)

Drug	Treatment	Mean ED_{50} µg/ml	95% Confidence Limits		Significance
			Lower	Upper	
Adrenaline	Control	0.70	0.76	0.59	P<0.001
	Reserpine	0.21	0.24	0.17	
Isoprenaline	Control	0.65	0.72	0.61	P<0.001
	Reserpine	0.21	0.24	0.15	
Phenylephrine	Control	7.3	7.7	7.1	P<0.001
	Reserpine	2.0	2.3	1.6	

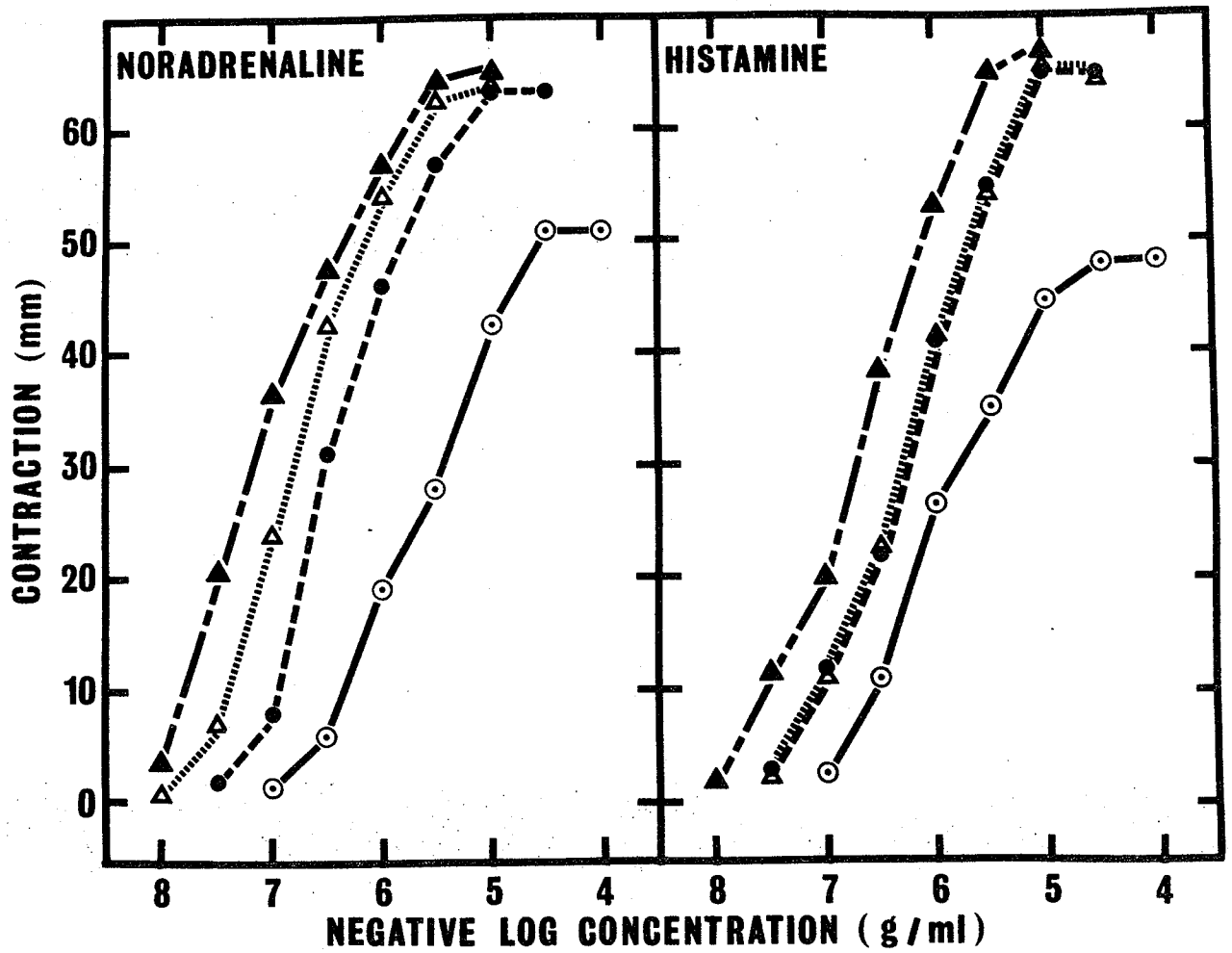


Fig. 10. Effect of reserpine (1 mg/kg) and cocaine (10^{-6} g/ml), (3×10^{-5} g/ml), on dose-response curves to noradrenaline and histamine in cat spleen.
Responses from normal control, O——O, are compared with responses from cats treated with reserpine, ●– –●, and with cocaine (10^{-6} g/ml), Δ····Δ, cocaine (3×10^{-5} g/ml), ▲— ·▲.

TABLE 15

POTENTIATION OF HISTAMINE AND NORADRENALINE BY RESERPINE (1 mg/kg) AND COCAINE (10^{-6} g/ml) AND (3×10^{-5} g/ml)

Drug	Treatment	Mean ED_{50} µg/ml	95% Confidence Limits		Significance
			Lower	Upper	
Histamine	Control	1.54	1.56	1.47	P<0.01
	Reserpine	0.69	0.77	0.53	
Histamine	Reserpine	0.69	0.77	0.53	N.S.
	Cocaine 10^{-6} g/ml	0.69	0.79	0.55	
Histamine	Reserpine	0.69	0.79	0.55	P<0.01
	Cocaine 3×10^{-5} g/ml	0.31	0.33	0.24	
Noradrenaline	Control	2.50	2.93	2.65	P<0.001
	Reserpine	0.57	0.64	0.51	
Noradrenaline	Reserpine	0.57	0.64	0.51	P<0.01
	Cocaine 10^{-6} g/ml	0.22	0.27	0.17	
Noradrenaline	Reserpine	0.57	0.64	0.51	P<0.001
	Cocaine 3×10^{-5} g/ml	0.086	0.095	0.081	

acetylcholine content of various tissues (Malhotra and Das, 1962; Green, Fleming and Schmidt, 1968).

Neither atropine nor phentolamine increased maximum responses significantly (Fig. 11). In addition measurements of length of strips after equilibration in the bathing fluid (see Method section, p.17) showed no significant difference between strips from untreated and reserpine treated animals which might be expected if there was a difference in tone between treated and untreated strips.

EFFECTS OF RESERPINE ON OTHER SMOOTH MUSCLE PREPARATIONS

To determine whether the unspecific potentiation and increase in maximum responses found after reserpine treatment in cat spleen was peculiar to spleen alone, we tested the effects of reserpine on guinea-pig ileum and rat uterus.

GUINEA-PIG ILEUM

Cumulative dose-response curves to acetylcholine, angiotensin and histamine were compared in sixteen experiments on segments of ileum from treated and untreated guinea-pigs. Only one agonist was tested per segment. Results and statistical analysis are shown in Fig. 12 and Tables 16 and 17.

Responses to all agonists tested were potentiated and maximum responses increased.

RAT UTERUS

Comparison of dose-response curves to acetylcholine, 5-hydroxy-tryptamine and oxytocin on segments of uteri from treated and untreated rats showed responses to agonist tested were potentiated and maximum responses increased.

Fig. 11.

Dose-response curves showing in A. The effect of atropine on responses to noradrenaline, in B the effect of phentolamine on responses to histamine.

A. Dose-response curves to noradrenaline.

O—O Control

●····● 90 min. later in same strip in presence of atropine 10^{-7} g/ml.

△····△ Dose-response curve in spleen strip from cat given reserpine 1 mg/kg.

●– –● Dose-response curve in presence of atropine 10^{-7} g/ml in same spleen strip from cat given reserpine.

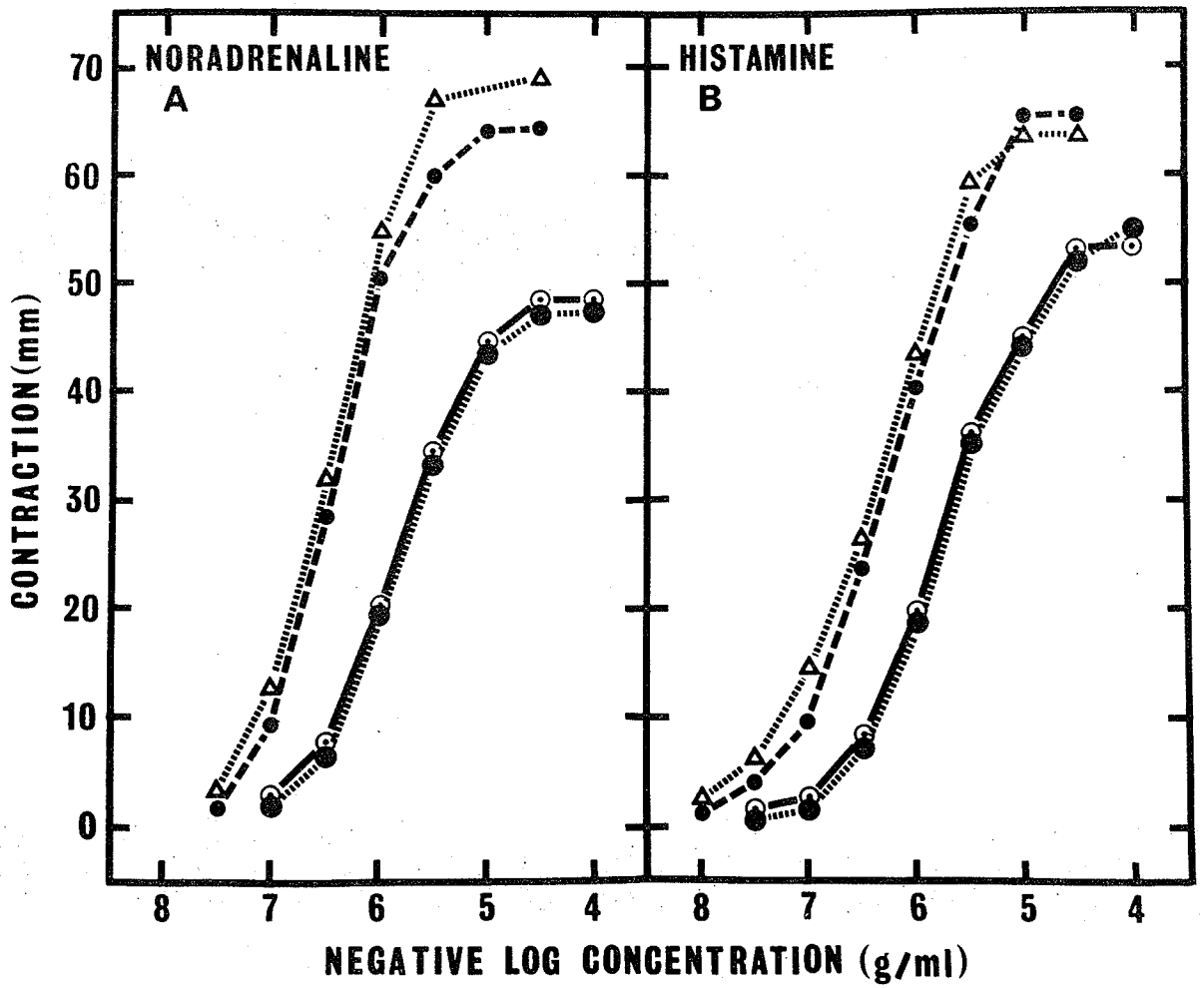
B. Dose-response curves to histamine.

O—O Control.

●····● Dose-response curve in a second strip from same spleen in presence of phentolamine 3×10^{-7} g/ml.

△····△ Dose-response curve in spleen strip from cat given reserpine 1 mg/kg.

●– –● Dose-response curve in presence of phentolamine 3×10^{-7} g/ml in a second strip from same spleen of cat given reserpine.


NORADRENALINE
A
HISTAMINE
B
CONTRACTION (mm)
70
60
50
40
30
20
10
0
8
7
6
5
4
NEGATIVE LOG CONCENTRATION (g/ml)

Results and statistical analysis of twelve experiments are shown in Fig. 13 and Tables 17 and 18.

EFFECT OF DENERVATION (14 DAYS) ON RESPONSES TO AGONISTS

Denervation is the classic technique of producing supersensitivity (Budge, 1855; Lewandowsky, 1899). Analysis of the results of these and many following reports are complicated in that experiments were done in vivo with many attending difficulties (see Introduction, p. 9).

Karr (1966) used a technique of stripping nerves from blood vessels entering the splenic capsule, but only tested responses to noradrenaline and therefore could not comment on the specificity of supersensitivity produced. Green and Fleming (1968) cut the postganglionic fibres of the splenic nerve near the spleen, but their technique was only partially successful in that analysis of denervated spleen showed that the catecholamine content had been reduced only to thirty per cent of control values. Moreover they also found variation in sensitivity between medial and lateral ends of spleens; cocaine significantly increased sensitivity of the lateral end, and they tested only noradrenaline.

With the procedure we used (See Methods, p.19) sensitivity changes were uniform throughout spleens. Comparisons of full dose-response curves to a variety of agents were tested.

CAT SPLEEN

In twenty-four experiments strips from spleens which had been denervated were compared with control strips. Full dose-response curves were obtained to acetylcholine, angiotensin, histamine and noradrenaline. Only one agonist was tested per strip.

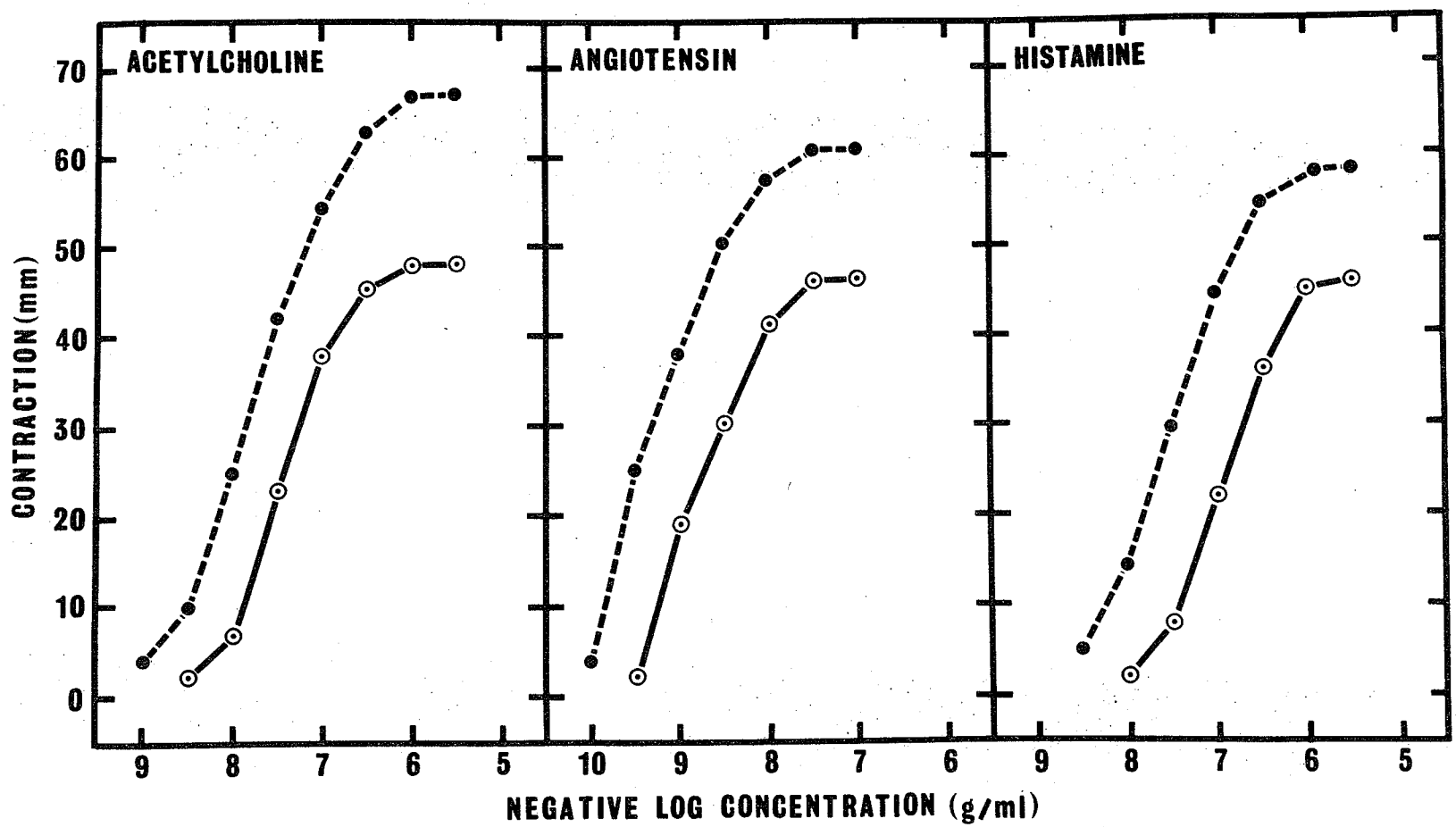


Fig. 12. Effect of reserpine pretreatment on dose-response curves to acetylcholine, angiotensin and histamine in guinea-pig ileum.
Responses from normal control ileum, O—O, are compared with responses of ileum from guinea-pigs pretreated with reserpine (1 mg/kg), ●- -●.

TABLE 16

POTENTIATION OF ACETYLCHOLINE, ANGIOTENSIN AND HISTAMINE BY RESERPINE (1 mg/kg) IN GUINEA-PIG ILEUM

Drug	Treatment	Mean ED_{50} μg/ml	95% Confidence Limits		Significance
			Lower	Upper	
Acetylcholine	Control	0.059	0.048	0.071	P<0.001
	Reserpine	0.019	0.015	0.024	
Angiotensin	Control	0.0018	0.0014	0.0023	P<0.005
	Reserpine	0.00067	0.00059	0.00077	
Histamine	Control	0.42	0.36	0.49	P<0.001
	Reserpine	0.11	0.09	0.13	

TABLE 17

EFFECT OF RESERPINE ON MAXIMUM RESPONSES TO AGONISTS IN GUINEA-PIG ILEUM AND RAT UTERUS

Agonist	Treatment	Maximum Responses Mean ± S.E.	Significance
	GUINEA-PIG ILEUM		
Acetylcholine	Control	49.11 ± 1.76	$P < 0.005$
	Reserpine	67.62 ± 1.92	
Angiotensin	Control	46.20 ± 1.13	$P < 0.005$
	Reserpine	60.70 ± 1.40	
Histamine	Control	45.80 ± 0.76	$P < 0.005$
	Reserpine	58.50 ± 1.38	
	RAT UTERUS		
Acetylcholine	Control	59.40 ± 0.76	$P < 0.005$
	Reserpine	70.13 ± 1.07	
5-Hydroxytryptamine	Control	55.20 ± 1.02	$P < 0.005$
	Reserpine	68.70 ± 2.05	
Oxytocin	Control	54.85 ± 1.26	$P < 0.005$
	Reserpine	69.95 ± 0.94	

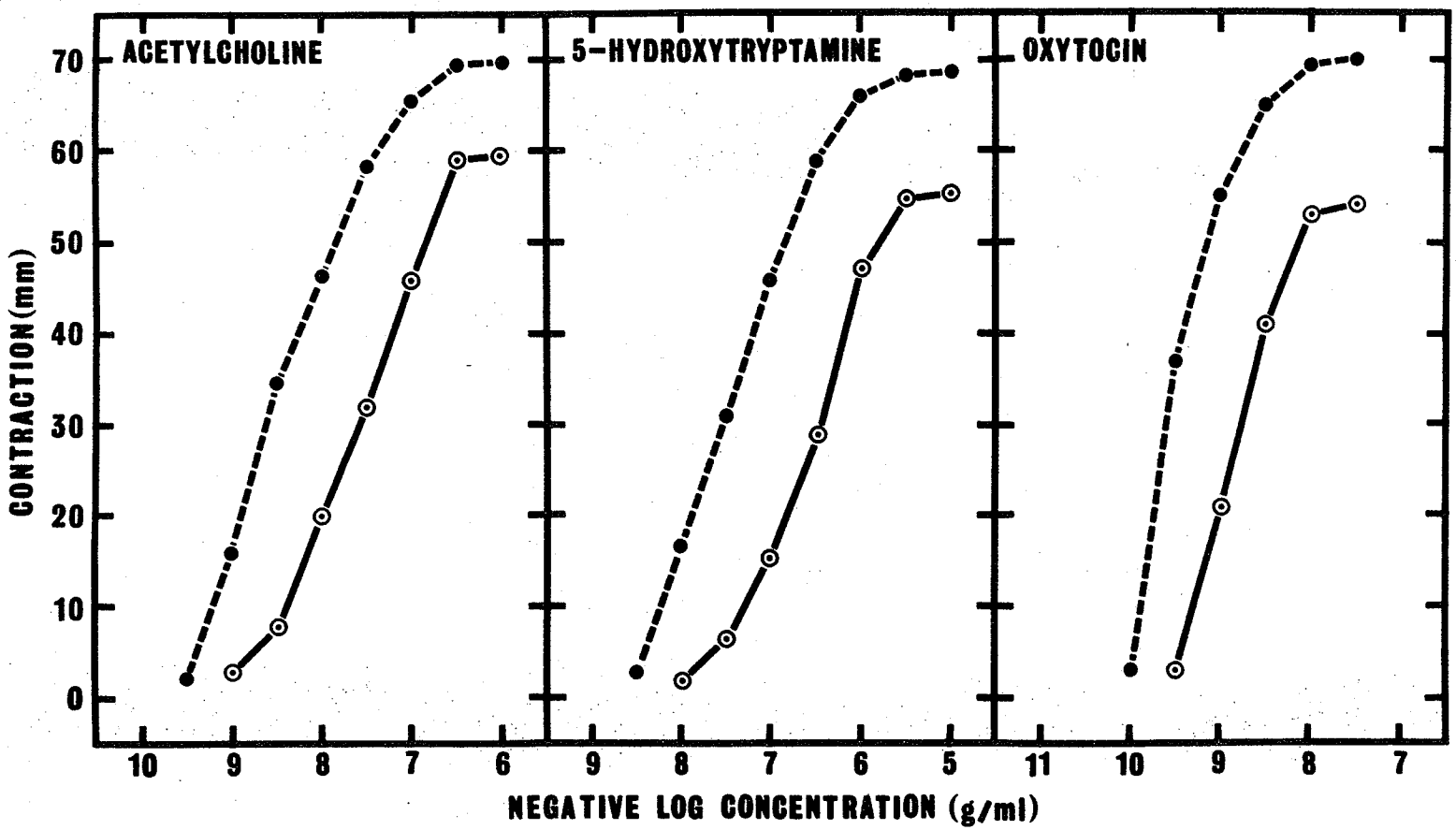


Fig. 13. Effect of reserpine pretreatment on dose-response curves to acetylcholine, 5-hydroxytryptamine and oxytocin in rat uterus.
Responses from normal control uteri, ○—○, are compared with responses of uteri from rats pretreated with reserpine (1 mg/kg), ●- -●.

TABLE 18

POTENTIATION OF ACETYLCHOLINE, 5-HYDROXYTRYPTAMINE AND OXYTOCIN BY RESERPINE (1 mg/kg)

Drug	Treatment	Mean ED_{50} µg/ml	95% Confidence Limits		Significance
			Lower	Upper	
Acetylcholine	Control	0.043	0.038	0.051	P<0.001
	Reserpine	0.005	0.004	0.0057	
5-Hydroxy-tryptamine	Control	0.50	0.42	0.55	P<0.001
	Reserpine	0.06	0.051	0.070	
Oxytocin	Control	0.0018	0.0013	0.0024	P<0.001
	Reserpine	0.00047	0.00037	0.00055	

Results and statistical analysis are shown in Fig. 14 and Table 19. Responses to all agonists tested were potentiated by denervation; maximum responses to agonists were unchanged.

RAT UTERUS

Dose-response curves to acetylcholine, angiotensin and 5-hydroxytryptamine were compared in twenty experiments on segments of uteri from denervated and control animals. Denervation potentiated responses to all three agonists; maximum responses were unchanged.

Results and statistical analysis are shown in Fig. 15 and Table 20.

DEPLETION AND UPTAKE OF CATECHOLAMINES

Various explanations have been put forward from time to time to account for the potentiating effect of cocaine, reserpine or of chronic denervation on the actions on a variety of tissues innervated by the sympathetic nervous system (see reviews by Furchgott, 1955; Trendelenburg, 1963, 1966). The currently popular hypothesis is that the concentration of a catecholamine in the extracellular fluid immediately in contact with the adrenergic receptors is normally limited by uptake into the adrenergic nerves and that interference of this uptake changes the concentration near the receptors. Accordingly we studied the effect of cocaine, denervation and reserpine on catecholamine content and uptake.

Catecholamine content of cat spleen, normal, denervated and reserpine treated is shown in Table 21. The effects of reserpine on catecholamine content of guinea-pig ileum, and reserpine or denervation on rat uterus are shown in Tables 22 and 23.

In each case reserpine or denervation significantly ($P<0.001$)

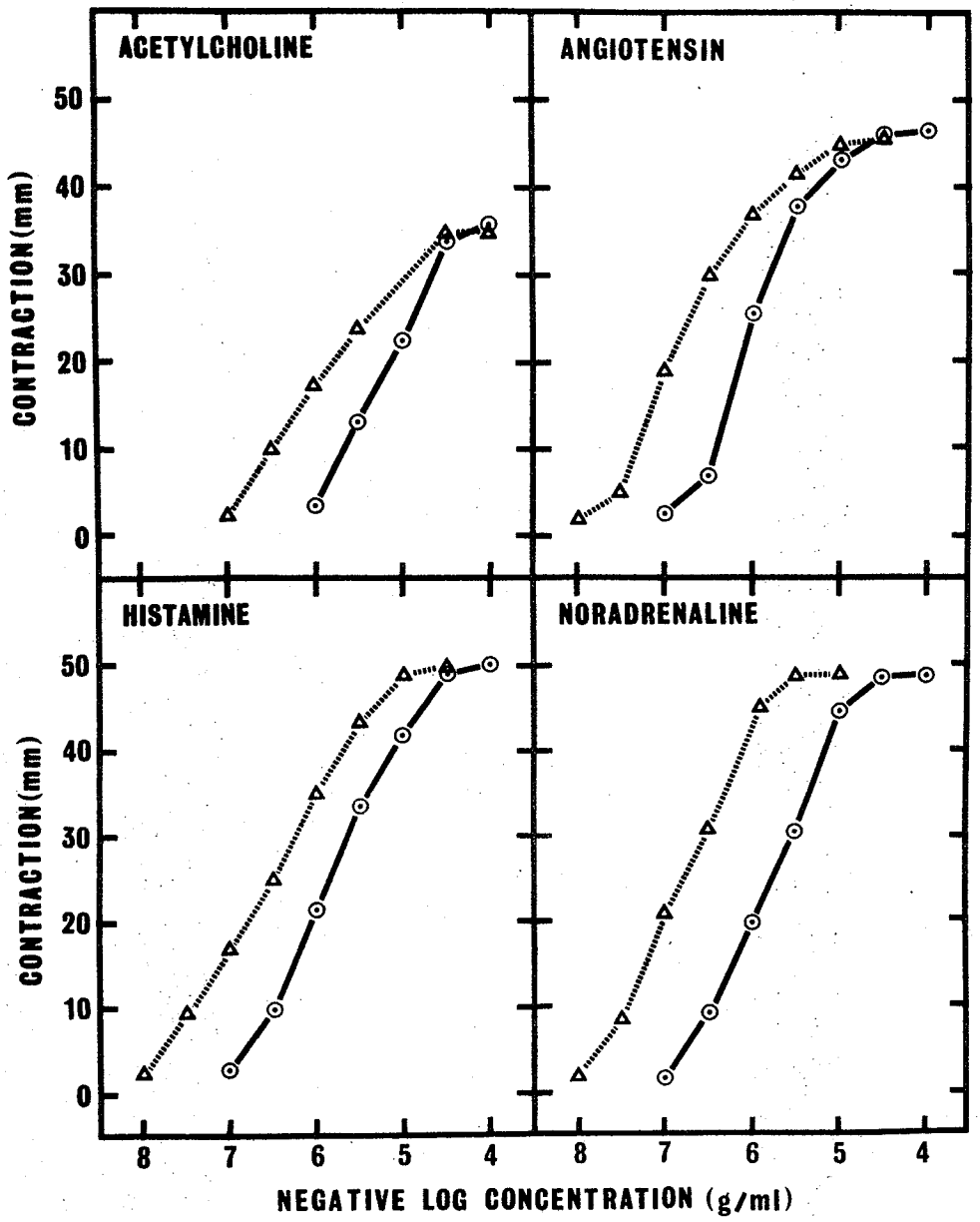


Fig. 14. Effect of denervation of cat spleen on dose-response curves to acetylcholine, angiotensin, histamine and noradrenaline. Responses from normal control spleen, O—O, are compared with responses from denervated spleen, Δ----Δ.

TABLE 19

POTENTIATION OF ACETYLCHOLINE, ANGIOTENSIN, HISTAMINE AND NORADRENALINE BY DENERVATION (14 DAYS)

Drug	Treatment	Mean ED_{50} $\mu g/ml$	95% Confidence Limits Lower	Upper	Significance
Acetylcholine	Control	7.7	7.1	8.5	$P<0.001$
	Denervation	1.0	0.91	1.13	
Angiotensin	Control	0.94	0.86	1.15	$P<0.001$
	Denervation	0.18	0.13	0.24	
Histamine	Control	1.47	1.29	1.67	$P<0.005$
	Denervation	0.50	0.41	0.62	
Noradrenaline	Control	2.4	2.11	2.65	$P<0.001$
	Denervation	0.20	0.17	0.26	

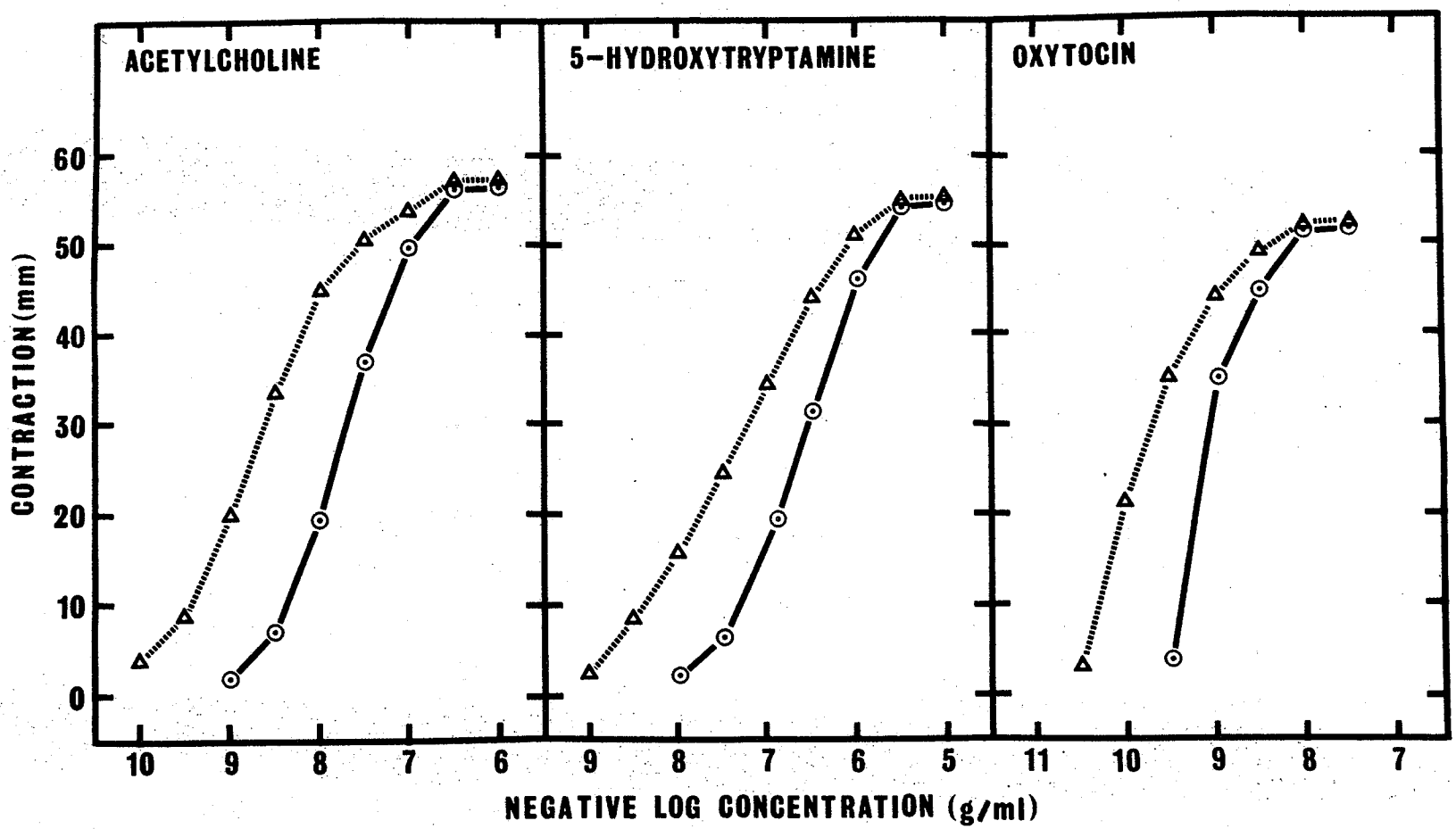


Fig. 15. Effect of denervation of rat uterus on dose-response curves to acetylcholine, 5-hydroxytryptamine and oxytocin.
Responses from normal control uteri, O—O, are compared with responses from denervated uteri, Δ····Δ.

TABLE 20

POTENTIATION OF ACETYLCHOLINE, 5-HYDROXYTRYPTAMINE AND OXYTOCIN BY DENERVATION (14 DAYS)

Drug	Treatment	Mean ED_{50} $\mu g/ml$	95% Confidence Limits		Significance
			Lower	Upper	
Acetylcholine	Control	0.023	0.028	0.017	P<0.001
	Denervation	0.003	0.0035	0.0027	
5-Hydroxy-tryptamine	Control	0.39	0.47	0.33	P<0.001
	Denervation	0.064	0.071	0.060	
Oxytocin	Control	0.00089	0.00097	0.00073	P<0.001
	Denervation	0.0018	0.00023	0.00011	

reduced the catecholamine content of treated tissues compared with controls. We noted that in cat spleen the degree of depletion of catecholamine content after treatment with reserpine was greater than that reported by other authors (Burn and Rand, 1959; Green and Fleming, 1968).

Examination of the other workers' papers showed that one possibility for the difference noted was that they used adult cats; (>2.0 kg) so we tested the effect of reserpine on catecholamine content of spleen and heart in two groups of animals (>2.0 kg) and (0.8 - 1.5 kg). Results of these experiments are shown in Table 24.

The catecholamine content of hearts and spleens of untreated control cats showed no significant difference between the two groups. Although reserpine significantly ($P<0.001$) depleted the catecholamine content of heart and spleen in both groups, the reduction in catecholamine content of both heart and spleen was significantly ($P<0.005$) greater in kittens (0.8 - 1.5 kg).

The failure of cocaine to enhance the action of isoprenaline on beta adrenergic receptors of various tissues (Raszkowski and Koelle, 1960; Stafford, 1963) is in accord with the uptake hypothesis, because uptake of isoprenaline is small (Hertting, 1964; Iversen, 1964). Isoprenaline, however, contracts the cat spleen by acting on alpha adrenergic receptors (Bickerton, 1963). We find that cocaine potentiates this action. This observation, in terms of the uptake hypothesis, is inconsistent with a low uptake of isoprenaline, so we have measured the uptake of isoprenaline by cat spleen and determined the effects of cocaine on it.

POTENTIATION OF ISOPRENALINE AND NORADRENALINE BY COCAINE

Cumulative dose-response curves to isoprenaline and noradrenaline

TABLE 21

COMPARISON OF CATECHOLAMINE CONTENT (μg/g TISSUE) OF CAT SPLEEN (0.8 - 1.5 kg)

	UNTREATED	RESERPINE PRETREATMENT (1 mg/kg)	DENERVATED (14 days)
	30 cats	34 cats	16 cats
	2.140	0.002	0.002
	5.096	0.002	0.002
	4.780	0.002	0.002
	3.750	0.002	0.002
	2.320	0.002	0.002
	1.771	0.002	0.002
	3.190	0.002	0.002
	2.510	0.002	0.002
	1.591	0.002	0.002
	3.369	0.002	0.002
	4.570	0.002	0.002
	2.901	0.002	0.002
	3.320	0.002	0.002
	1.787	0.002	0.002
	5.285	0.002	0.119
	3.785	0.002	0.024
	1.870	0.002	
	4.793	0.002	
	1.630	0.002	
	2.069	0.002	
	2.159	0.002	
	2.860	0.002	
	4.272	0.002	
	5.139	0.002	
	1.364	0.002	
	1.603	0.002	
	1.580	0.002	
	1.589	0.002	
	2.290	0.002	
	1.407	0.002	
		0.009	
		0.012	
		0.050	
		0.120	
Mean ± S.E.	2.893 ± 0.236	0.0056 ± 0.0038	0.0089 ± 0.006

TABLE 22

CATECHOLAMINE CONTENT (μg/g TISSUE) OF GUINEA-PIG ILEUM

	Untreated	Reserpine Pretreatment (1 mg/kg)
	0.532	0.051
	0.492	0.071
	0.452	0.086
	0.449	0.014
	0.510	0.042
	0.473	0.039
Mean ± S.E.	0.484 ± 0.013	0.050 ± 0.010

TABLE 23

CATECHOLAMINE CONTENT (μg/g TISSUE) OF RAT UTERUS

	Untreated	Reserpine Pretreatment (1 mg/kg)	Denervated (14 days)
	0.579	0.034	0.004
	0.492	0.003	0.009
	0.731	0.031	0.013
	0.511	0.014	0.006
	0.493	0.009	0.000
	0.626	0.019	0.017
Mean ± S.E.	0.572 ± 0.038	0.018 ± 0.004	0.008 ± 0.002

TABLE 24

COMPARISON OF EFFECT OF RESERPINE (1 mg/kg) ON CATECHOLAMINE CONTENT (μg/g TISSUE) OF CAT HEART AND SPLEEN BETWEEN ADULT (>2.0 kg) AND KITTEN (<1.5 kg)

	HEART		SPLEEN	
	UNTREATED	RESERPINE	UNTREATED	RESERPINE
Adult	1.596	0.113	1.990	0.133
	2.027	0.267	2.670	0.071
	3.756	0.184	2.440	0.114
	1.318	0.120	3.190	0.226
	1.845	0.143	2.210	0.164
Mean ± S.E.	1.71 ± 0.12	0.17 ± 0.03	2.34 ± 0.23	0.15 ± 0.03
Kitten	2.200	.002	2.140	.002
	1.808	.002	5.096	.002
	1.803	.002	1.780	.002
	2.075	.002	2.860	.002
	1.940	.002	2.320	.002
Mean ± S.E.	1.97 ± 0.08	.002	2.85 ± 0.42	.002

before and after cocaine (10^{-5} g/ml) were compared in five experiments on spleen strips from normal cats and six experiments on strips from cats given reserpine (1 mg/kg intraperitoneally) twenty-four hours before the experiment. Four strips from the same spleen were used in each experiment; isoprenaline and noradrenaline were each tested on two strips, one strip before and after cocaine, the second strip as a time control without cocaine. In both series of experiments cocaine potentiated both isoprenaline and noradrenaline, while the time control dose-response curves were unchanged (Fig. 16 and 17). Potentiation of isoprenaline was significantly less than that of noradrenaline ($P<0.001$). Statistical analysis of the results is shown in Tables 25 and 26.

UPTAKE OF ISOPRENALINE AND NORADRENALINE BY CAT SPLEEN

Strips from denervated cat spleen or from cats pretreated with reserpine were exposed to a loading dose of isoprenaline or noradrenaline (10^{-4} g/ml) for five minutes and then washed until the strips had relaxed. Other strips from the same animals were not exposed to the agonists and served as controls. All strips were then taken from the organ baths; the control tissues were assayed for total catecholamines and treated tissues for isoprenaline and noradrenaline respectively. Isoprenaline and noradrenaline were taken up by spleen in each of thirteen experiments from reserpine treated cats. The isoprenaline contents were 2.19 ± 0.28 μg/g tissue, and noradrenaline content was 2.27 ± 0.34 μg/g tissue.

Spleens previously denervated showed a greatly reduced uptake; the values for isoprenaline and noradrenaline were 0.14 ± 0.02 and 0.17 ± 0.03 μg/g respectively. Total catecholamine content of unloaded strips from denervated and reserpine treated strips was less than 0.002 μg/g tissue.

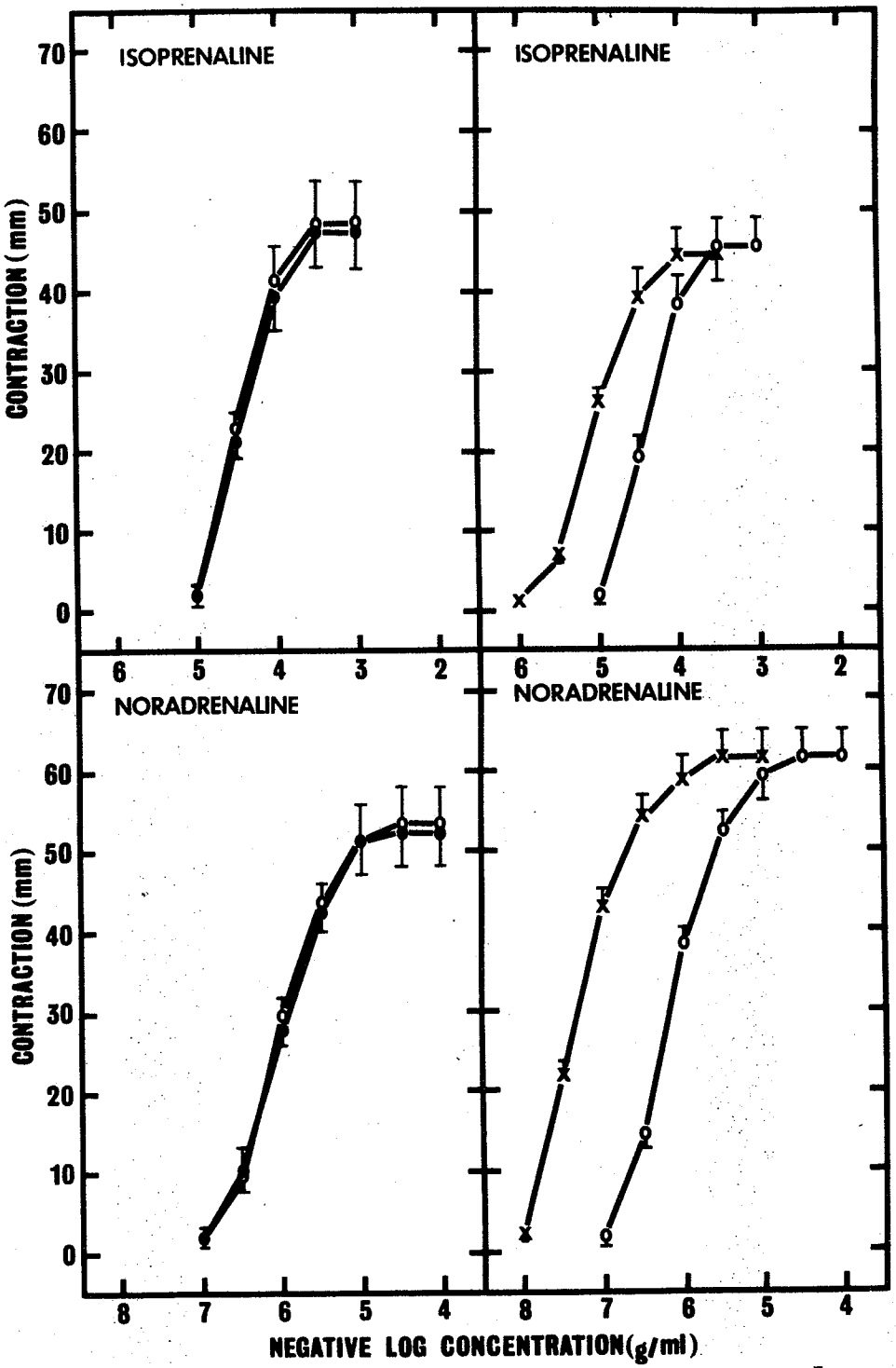


Fig. 16. Potentiating effect of cocaine (1×10^{-5} g/ml) on isoprenaline and noradrenaline effects in spleen strips from normal cats. Responses from normal control spleen, O—O, are compared with responses with cocaine, X—X, and time controls, ●—●.

TABLE 25

POTENTIATION OF ISOPRENALINE AND NORADRENALINE BY COCAINE (10^{-5} g/ml)

Drug	Treatment	Mean ED_{50} $\mu g/ml$	95% Confidence Limits		Significance
			Lower	Upper	
Isoprenaline	Control	36.6	33.6	40.0	N.S.
	Time	39.5	36.2	43.1	
Noradrenaline	Control	0.99	0.86	1.14	N.S.
	Time	1.02	0.89	1.17	
Isoprenaline	Control	41.0	33.6	50.1	P<0.001
	Cocaine	8.7	7.1	10.7	
Noradrenaline	Control	0.84	0.62	1.12	P<0.001
	Cocaine	0.07	0.05	0.09	

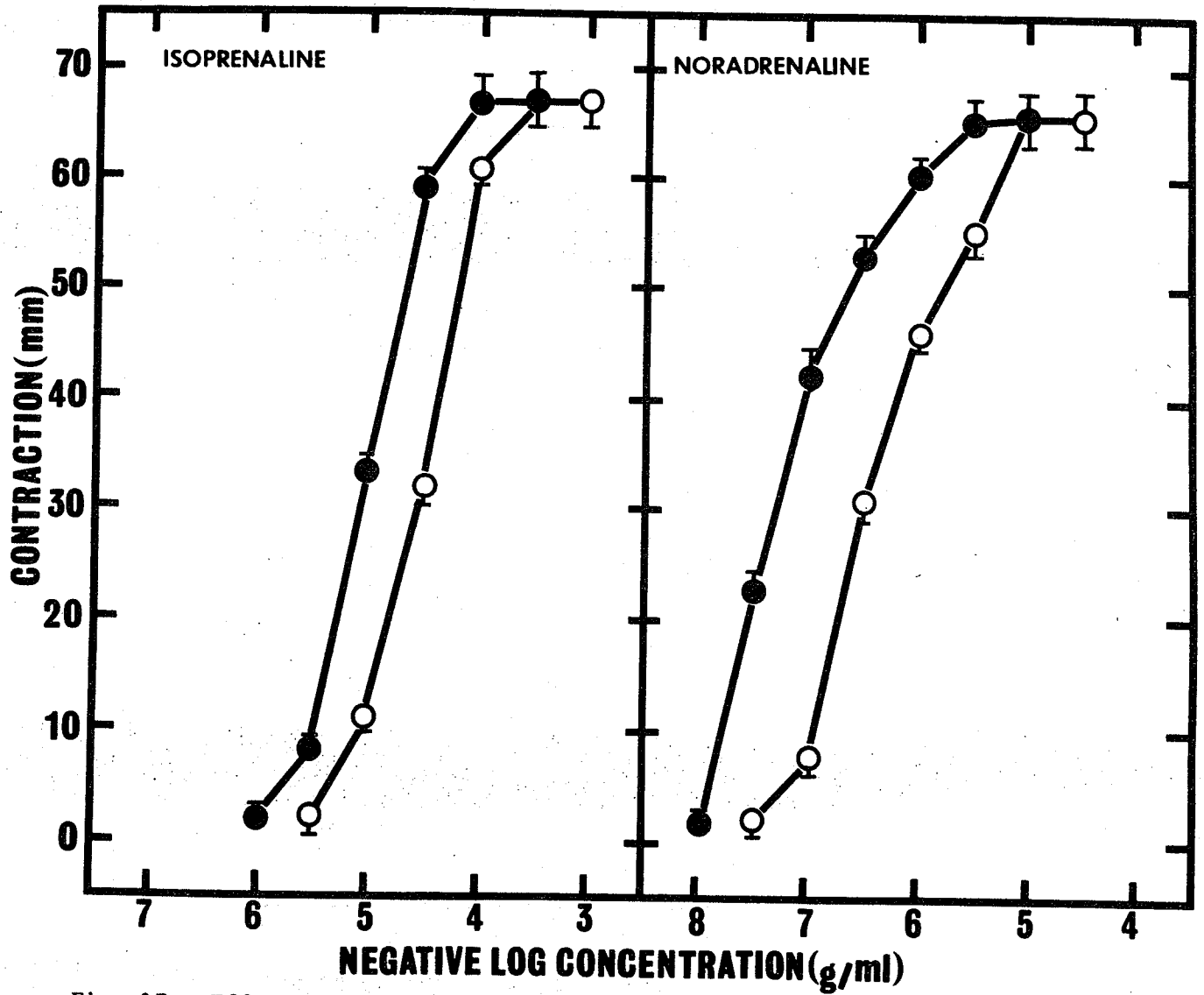


Fig. 17. Effect of cocaine on dose-response curves to isoprenaline and noradrenaline in spleen from cats pretreated with reserpine (1 mg/kg). O—O, Before Cocaine, ●——●, After Cocaine, 10^{-5} g/ml.

TABLE 26

POTENTIATION OF ISOPRENALINE AND NORADRENALINE BY COCAINE (10^{-5} g/ml) AFTER RESERPINE

Drug	Treatment	Mean ED_{50} μg/ml	95% Confidence Limits		Significance
			Lower	Upper	
Isoprenaline	Control	28.8	26.7	31.0	P<0.001
	Cocaine	9.7	9.0	10.4	
Noradrenaline	Control	0.57	0.50	0.58	P<0.001
	Cocaine	0.10	0.09	0.11	

EFFECT OF COCAINE ON UPTAKE OF ISOPRENALINE AND NORADRENALINE

The effects of cocaine on uptake of isoprenaline and noradrenaline were tested in eighteen experiments. For each experiment five strips from the same spleen were used. In all cases the cats were given reserpine (1 mg/kg intraperitoneally) before the experiment. Two strips were exposed to a loading concentration of isoprenaline, in one strip in the presence of cocaine (10^{-5} g/ml) applied five minutes before isoprenaline, in the second without cocaine. Two strips were similarly exposed to noradrenaline with or without cocaine. The fifth strip was exposed to cocaine only and served as a control. Loading concentrations of isoprenaline or noradrenaline were 10^{-7}, 10^{-6} and 10^{-5} g/ml. Strips were exposed to the catecholamines for five minutes, washed till the contraction had disappeared, and then assayed for isoprenaline and noradrenaline content. Cocaine significantly reduced the uptake of noradrenaline but did not alter the uptake of isoprenaline (Table 27.)

TABLE 27

EFFECT OF COCAINE (10^{-5} g/ml) ON UPTAKE OF ISOPRENALINE AND NORADRENALINE IN SPLEEN STRIPS FROM RESERPINE TREATED CATS.

Loading Drug	Cocaine	Tissue Content g/g tissue — Loading Concentration			
		10^{-7} g/ml	10^{-6} g/ml	10^{-5} g/ml	0
Noradrenaline	0	0.35 ± 0.03	0.56 ± 0.07	1.40 ± 0.10	<0.002
Noradrenaline	10^{-5} g/ml	0.06 ± 0.02	0.17 ± 0.05	0.54 ± 0.26	
		P<.001	P<0.005	P<0.001	
Isoprenaline	0	0.25 ± 0.03	0.32 ± 0.06	0.96 ± 0.02	
	10^{-5} g/ml	0.25 ± 0.04	0.31 ± 0.07	0.95 ± 0.03	
		N.S.	N.S.	N.S.	

SECTION IV

DISCUSSION

DISCUSSION AND CONCLUSIONS

The major part of this study was done on cat spleen. Accordingly we began by examining some basic properties of cat spleen which could enable us to determine where changes might have occurred after production of supersensitivity.

Preliminary experiments agreed with Innes (1962) that cat spleen was contracted by various biogenic amines and extended his findings to full dose-response curves to acetylcholine, angiotensin, histamine and various sympathomimetic amines. The findings that spleen strips from kittens (0.8 - 1.5 kg) showed less variation in responses to agonists and a greater duration of viability as measured by sodium and potassium content is not really surprising in that *in vitro* preparations are mainly dependent on diffusion for metabolic requirements and the thickness of strips from adult cats was significantly greater ($P<0.005$).

Other factors than diffusion may play a role in the difference between adults and kittens and differences in sensitivities of young and adult animals have been shown to exist *in vivo* (Schlossman, 1937; Fouts, 1962; Kupferberg and Way, 1963; Spain, 1963). These authors have shown that *in vivo* some of the difference between young and adult animals were explained by differences in ability to metabolize drugs or by differences in their distribution.

MAXIMUM RESPONSES TO AGONISTS

The results obtained by producing the maximum response to one agonist and a superimposed response by addition of a second class of agonist clearly showed that spleen strips are capable of further contrac-

tile response than that produced by one class of agonist, and that the limitation on maximum responses to the drugs tested is not the contractile elements per se, in control tissues or tissues made supersensitive by cocaine, denervation and reserpine. Kuenzle (1960) proposed this technique would be useful for confirming the existence of specific drug receptors in a tissue and was able to show specific receptors for acetylcholine, histamine and adrenaline in rabbit aorta. The main criticisms of this technique were that contractility or desensitization might be interfering factors, or that a compound with greater intrinsic activity could produce a further response by successful competition for the receptors with which the first drug has combined, thus leading to a false conclusion of separate receptors for two agonists.

The observation that tissues gave a further response on removal of the stimulus of an agonist at a concentration producing the maximum response obtainable by that drug, suggested that some desensitization effect was occurring; stretch, pH and temperature changes were carefully excluded. This desensitization appeared to be relatively specific for each agonist, in that a second agent produced a further tissue response, which would not be expected if unspecific desensitization generally produced by high concentrations of an agonist had occurred (Cantoni and Eastman, 1946).

A possible explanation for this phenomena is that inhibitory receptors exist with which agonists combine. These are not the beta adrenergic receptors, since the phenomenon still occurs in the presence of a beta receptor antagonist, and it is not confined to adrenergic drugs; all other classes of agonist tested produce the phenomenon. The phenomenon

can be readily explained if the affinity and intrinsic activity of agonists for the inhibitory receptors are small compared to the excitatory drug receptors. The observed agonist response will be an arithmetic mean of activation of both sets of receptors. When the agonist is removed from the bathing fluid the inhibitory drug-receptor complex will quickly dissociate due to the low affinity, the excitatory drug-receptor complex will now be able to produce its full effect. If the agonists have also a relatively low intrinsic activity when combined with this inhibitory receptor, this would explain why near maximum doses of agonist are required before there is any effect on the normal excitatory response.

RECEPTOR RESERVE, AFFINITY AND INTRINSIC ACTIVITY OF SYMPATHOMIMETIC AMINES IN CAT SPLEEN.

An important aspect of drug action is the quantitative relation between the concentration of agonist in the vicinity of the receptors and the resulting effect. This relation can be studied by means of determination of the dose-effect curve for a drug in a simplified biological system. Classical receptor theory postulated that the response of a given organ or tissue is proportional to the number of receptors occupied by the stimulating agent and that the maximal response occurs when all receptors are occupied. The findings of Nickerson (1956) and Stephenson (1956) that the maximal response to histamine in guinea-pig ileum required occupancy of only a portion of the available receptors suggested that theoretical deductions from mass action analysis of dose-response curves should be accepted with caution. These findings found ready acceptance because there were unexplained anomalies in drug receptor interactions, and because of the complex nature of tissue responses.

The finding of spare receptors gained such acceptance that everyone assumed without adequate studies that all tissues had spare receptors, until 1966 when Offenheimer and Ariens reported rat stomach strips had no spare 5-hydroxytryptamine receptors and Lewis and Miller showed rat seminal vesicle had no spare adrenergic receptors.

Our results showing no spare adrenergic receptors in cat spleen and those of Cook (1970) who reported that there was no histamine receptor reserve in rabbit aorta add to the growing evidence that in many tissues the limiting factor in determining the magnitude of tissue response may well be receptor occupancy. This has not yet been appreciated by the scientific community, although the only tissues where a receptor reserve has been reported is guinea-pig ileum and human fetal ileum (Boreus, 1968). We have shown that contractile capability of spleen is not the limiting factor with the agents tested and the family of dose-response curves of sympathomimetic amines (Fig. 6) could now not be explained by variations in the percentage of available receptors occupied. pA_3 values agreed that these drugs acted via a common receptor.

Attempts to estimate affinity of various drugs have invariably involved theoretical calculations from equations where one assumption in their derivation has been the existence of a receptor reserve, although in many cases this was valid, since the experimental tissue used was guinea-pig ileum. Barlow et al., 1965; Waud, 1969; Tallarida et al., 1970). The use of receptor protection technique in a tissue where no receptor reserve exists provides a direct method for measuring and comparing affinities of different drugs without recourse to calculations which are based on many unproved assumptions. While variation between

experiments does not allow exact quantification of affinities, a relative measure of affinity is provided.

In a family of dose-response curves the position of the dose-response curve of any one agonist, is determined by affinity, intrinsic activity and the number of available receptors. Where there is no tissue receptor reserve, as we found for sympathomimetic amines in cat spleen, all full agonists have the same intrinsic activity and position of their dose-response curves should be determined by relative affinities.

The results of our experiments on measuring affinity (Table 9) show general agreement with predictions that were made that the position of the response-curve to a full agonist would be determined by the affinity of the agonist, changes in affinity and intrinsic activity would determine the position of response-curves to partial agonists. The only anomalous result was that of tyramine where the measured affinity was much higher than that expected from the position of the dose-response curve. This result is, however, readily explained in view of the fact that tyramine exerts its major effects in cat spleen via noradrenaline, released from catecholamine storage sites (Innes, 1962), and measurements of affinity of indirect or mixed acting agents should be interpreted with caution. The measurements could be made in tissues depleted of catecholamines, but again caution would be essential, since such treatment could produce tissue changes affecting affinity measurements.

The criticisms of Waud (1962) on the use of protection technique were that huge doses of an agonist used for protection of receptors, possibly could protect other receptors than those normally occupied by an agonist and thus lead to erroneus conclusions, when receptor protection

was used to differentiate receptors. However we never exceeded the dose of an agonist required to produce a maximum response and compared only responses of the same agonist before and after phenoxybenzamine blockade. The comparisons we made were of the ability of agonists which act on the same receptors, to competitively protect these receptors against phenoxybenzamine blockade. This use of protection technique is eminently suitable for measuring affinity in tissues where there is no receptor reserve. Responses were compared only at maximum responses where in both cases there was 100% receptor occupancy.

Classical approaches to drug-receptor interactions postulate that partial agonists besides possessing a lower intrinsic activity and occupying, with their maximally effective dose, all available receptors for that class of drugs, should also possess higher affinities than full agonists. Indeed a recognized test of partial agonism (Ariens et al., 1956) was based on the findings that a partial agonist when tested with a full agonist would prevent the full agonist from producing the maximal effect through competition for the available receptors. Our results on cat spleen show that phenylephrine, a partial agonist, does not have higher affinity than adrenaline, noradrenaline or nordefrine, all full agonists.

Ariens et al. (1956) used guinea-pig ileum in their experiments on partial agonism and our affinity measurements suggest either that caution should be used in interpreting results based on drug receptor interactions in tissues where there is a receptor reserve or that our measure of affinities is not correct. This demands further investigation and we propose to apply the technique of Ariens et al. using cat spleen.

This will provide evidence that will either justify our method of measuring affinity or stimulate further work to explain these anomalies.

POTENTIATION OF RESPONSES TO AGONIST BY COCAINE

Many workers attempt to justify their acceptance of the uptake hypothesis as the sole explanation of the potentiating action of cocaine by quoting reports which state cocaine does not potentiate responses to agonists other than catecholamines, though there are reports that cocaine does potentiate responses to other classes of agonists (Rosenblueth, 1932; Innes and Kosterlitz, 1954; Kalsner and Nickerson, 1969). Recently many proponents of the uptake hypothesis have used the term presynaptic changes to describe the role of cocaine in production of supersensitivity and post-synaptic changes to account for the supersensitivity observed after reserpine or denervation. These terms, as used by these workers, imply that cocaine produces only a supersensitivity to catecholamines by an action on sympathetic nerve endings, whereas the other procedures produce changes in the effector cells such that an unspecific supersensitivity is observed.

Trendelenburg (1965) and Green and Fleming (1967, 1968) use these terms, and a major criticism of their interpretations is that their criteria for specificity of supersensitivity is based on changes in pD_2 values of phenoxybenzamine against noradrenaline with no change in pA_2 values of phentolamine against noradrenaline. These authors state that a causal relationship exists between changes in pD_2 values and unspecific supersensitivity. This has never been established and is strongly reminiscent of the arguments these and many other workers have used: namely that as changes in uptake accompanied supersensitivity there must be a

causal relationship between them.

As pA_2 and pD_2 are both measures of antagonism at the receptor level, the difference being that in pA_2 determinations a competitive antagonist is used while in pD_2 determinations a non-equilibrium antagonist is used, the extrapolation of changes in pD_2 values reflecting post-receptor events seems completely unjustified. The obvious test for specificity of supersensitivity is to determine responses to agonists which act on different receptors and determine whether a procedure does produce supersensitivity and if so the specificity produced. Fleming (1968) showed that chronic ganglionic blockade produced unspecific supersensitivity in guinea-pig ileum; responses to acetylcholine, histamine, 5-hydroxytryptamine and potassium were potentiated but he did not check if pD_2 values were changed.

Our results clearly show that cocaine produced both specific and unspecific supersensitivity in cat spleen. Cocaine 10^{-8} - 10^{-5} g/ml potentiated only responses to catecholamines, whereas cocaine 3×10^{-5} g/ml produced maximum potentiation of catecholamine responses and potentiated responses to acetylcholine, angiotensin and histamine. Green and Fleming (1968) studied supersensitivity in cat spleen and stated that cocaine produced a specific supersensitivity, yet they tested only noradrenaline responses. As the concentration of cocaine used by these workers was 10^{-5} g/ml they would have arrived at erroneous conclusions on the ability of cocaine to produce unspecific supersensitivity even if they had tested this by the use of different classes of agonists. The ability of cocaine to produce unspecific supersensitivity is not limited to cat spleen as 3×10^{-5} g/ml potentiates responses to acetylcholine, angiotensin and

5-hydroxytryptamine in rat uterus (Innes, unpublished data).

Barnett et al. (1968) reported that cocaine and other agents which interfere with neuronal uptake of noradrenaline increased the maximum responses to noradrenaline in rat vas deferens. They attributed this to a decrease in auto-inhibition of noradrenaline responses. Kasuya and Goto (1968) also reported that cocaine increased maximum responses to a variety of agonists in rat vas deferens and decreased the threshold of calcium required for contraction. These workers concluded that cocaine can produce a specific supersensitivity to catecholamines and unspecific supersensitivity probably involving utilization of calcium at a post-receptor level.

We agree with Karr (1966) and Reiffenstein (1968) that cocaine does not change maximal responses to noradrenaline in cat spleen and have extended these findings to a variety of sympathomimetic amines and to acetylcholine, angiotensin and histamine. Our experiments on maximal responses and receptor reserve showed that the limiting factor in maximal drug responses in spleen sensitized by cocaine is not contractile capability and that the observed supersensitivity can not be by an increase in the number of available receptors.

That changes in affinity of the alpha adrenergic receptor might be produced by cocaine and thus explain the observed supersensitivity was first suggested by Clark (1937). The results of our determinations of pA_3 values with and without cocaine at concentrations of 10^{-6} and 3×10^{-5} g/ml showed no change in pA_3 values. These determinations were made with phentolamine as the antagonist; the agonists tested were sympathomimetic amines, both full and partial agonists, and histamine as a class of

agonist acting on different receptors. We interpret this to mean that no change in affinity of alpha adrenergic or histamine receptors was produced by cocaine. Although this interpretation can be criticized on the grounds that affinity of the alpha adrenergic receptors changed equally for agonists and antagonist, this is rather unlikely in the case of the histamine receptor because phentolamine is not a competitive antihistaminic agent. pA_3 values of phentolamine against histamine or acetylcholine are very similar, 4.32 and 4.19 respectively; compared with pA_3 values against the sympathomimetic amines tested this reflects a three hundred times increase in the concentration of phentolamine required to cause the same degree of antagonism and is indicative of an unspecific antagonism probably at a post-receptor level. Changes in affinity of receptors caused by cocaine at a concentration of 3×10^{-5} g/ml which produces an unspecific supersensitivity would therefore probably still be detected.

SUPERSENSITIVITY PRODUCED BY RESERPINE

Although there is general agreement among authors that reserpine can produce supersensitivity (Innes, 1960; Trendelenburg and Weiner, 1962; Westfall and Fleming, 1968; Kalsner and Nickerson, 1969; Davidson and Innes, 1970) there is still a dispute on the specificity of supersensitivity produced and the duration of pretreatment required. Two main schools of thought exist. One maintains that reserpine produces supersensitivity within twenty-four hours, the second maintains that a minimum of seventy-two hours treatment is required. Disagreement on the specificity of supersensitivity observed occurs in both schools. Explanations for differences in results between various workers might be due to differences

in the dose of reserpine given; (quoted amounts vary from 0.1 mg/kg to 5 mg/kg daily), species and organ variation, in sensitivity, and routes of administration and metabolism.

The dose of reserpine chosen for our experiments was 1 mg/kg given intraperitoneally and animals were killed twenty-four hours later. Though guinea-pigs and rats tolerated doses of reserpine of 5 mg/kg, with such doses less than 5 per cent of cats of 0.8 to 1.5 kg survived. Authors who reported experiments where they treated cats with doses of 5 mg/kg of reserpine did not comment on the percentage of cats surviving and invariably used adult cats (>2.0 kg).

We noted that the depletion of splenic catecholamines we obtained was greater than that reported by other authors (Weiner and Trendelenburg, 1962). The results of our experiments where we compared the effects of reserpine (1 mg/kg) on catecholamine depletion in adult and young cats showed that although there was no significant difference in normal endogenous catecholamine content between the two age groups in the organs studied, namely heart and spleen, reserpine produced a greater depletion ($P<0.001$) of catecholamines in both organs in young cats.

Iversen *et al.* (1967) reported that ability to accumulate ^{3}H noradrenaline in rat heart and spleen developed parallel to the endogenous noradrenaline content, but they did not comment on the ability of these organs to retain catecholamines; our results are in contrast because we found no significant difference in endogenous catecholamine content between the two groups in the organs we studied. Privitera *et al.* (1969) reported no differences in the sensitivities of the cardiovascular systems of newborn and adult dogs to various biogenic amines, and it is

of particular interest that pressor and chronotropic responses to tyramine were comparable, which suggested that newborn dogs were as capable of releasing catecholamines from adrenergic nerve endings as the adult animals. Results of Kulkorni and Shideman (1966) are extremely interesting. They found that there were significant differences in catecholamine content of rat brains between different age groups. At 11 days of age, the brain catecholamine content was approximately thirty per cent of that in the adult. A gradual increase in catecholamine content occurred with increasing age till at fifty days catecholamine content was approximately ninety per cent of that in adults.

The effects of varying doses of reserpine (0.1 - 1.5 mg/kg) were studied in brain catecholamine content and they found that at all dose levels of reserpine tested, the rate of catecholamine depletion was always significantly greater in the young animals and that rate of recovery of catecholamine content was significantly greater in adults. The catecholamine content of adult rat brain four days after administration of reserpine (1 mg/kg) did not differ significantly when compared with untreated rats. Even ten days after administration of the same dose of reserpine the catecholamine content of young rat brains was significantly less than that of untreated littermate controls.

Although earlier workers postulated a "hit and run" effect of reserpine to explain the mechanism of action, mainly because they could not detect any reserpine present in tissues while catecholamine depletion lasted several days, newer chemical methods have established that the biological half-life of reserpine can be as long as 12 days (Maass et al. 1969; Wagner and Stitzel, 1969). The reason for the difference in sensi-

tivity to reserpine between young and adult cats is not clear but is most likely due to differences in ability to metabolize the drug. Such differences in sensitivities of young and adult animals for other drugs have been explained by differences in ability to metabolize drugs (Fouts, 1962; Jardorf et al., 1958).

We found that reserpine treatment caused supersensitivity in all species tested and the required depletion of catecholamines for experiments on catecholamine uptake (Davidson and Innes, 1970). Treatment with reserpine potentiated responses to acetylcholine, angiotensin, histamine and noradrenaline in cat spleen, as well as responses to other sympathomimetic amines, both full and partial agonists.

This unspecific supersensitivity was also produced by reserpine in guinea-pig ileum and rat uterus, where responses to acetylcholine, angiotensin, histamine, and acetylcholine, 5-hydroxytryptamine and oxytocin respectively were potentiated.

The lack of specificity of supersensitivity produced by reserpine treatment was consistent for different organs and species. A most interesting finding was an increase in maximum responses to all agonists tested in each organ. Our experiments on spare receptors and maximum responses in cat spleen showed that 100% receptor occupancy was still necessary to obtain maximum responses in spleen strips from reserpine treated animals and that these tissues were still capable of further contractile response. However we cannot exclude the possibility that there had been an increase in the number of available receptors in reserpine treated spleen and that this caused the increase in maximum response. This explanation is considered less likely in that maximum responses to

acetylcholine and histamine were also increased in guinea-pig ileum where there already was a receptor reserve for these agonists. Changes in tone of strips of spleen from reserpine treated cats due to alteration of neurohumoral influences could have been responsible for the increase in maximum responses to agonists. Experiments where we attempted to produce such changes in maximum responses by the use of phentolamine and atropine, antagonists of noradrenaline and acetylcholine respectively, showed that no changes occurred. This and the fact that length of spleen strips from reserpine or untreated cats were not significantly different suggested that changes in tone were not responsible for the increased maximum responses.

The finding that cocaine 10^{-6} g/ml specifically potentiated responses to catecholamines while cocaine 3×10^{-5} g/ml potentiated responses to noradrenaline and histamine in spleen strips from untreated or reserpine treated cats suggested that either they produced supersensitivity by different mechanisms or that cocaine was still capable of further activation of a common mechanism. The findings of Davidson and Innes (1969) that pretreatment of cats with cocaine (10 mg/kg) before treatment with reserpine resulted in supersensitivity equivalent to that produced by reserpine treatment plus cocaine given "in vitro", but prevented the increase in maximum responses normally found after reserpine treatment, suggests that cocaine and reserpine have some common points of action.

The findings of unspecific supersensitivity and increased maximum responses, and the fact that for sympathomimetic amines no changes could be detected in their affinity for the adrenergic receptor in reser-

pine treated cats (Karr, 1966) strongly suggests that reserpine produces supersensitivity by altering post-receptor events in excitation-contraction possibly at some common final pathway.

DENERVATION SUPERSENSITIVITY

The results of our experiments show clearly that in cat spleen and rat uterus the "law of denervation" (Cannon, 1939) held true, in that unspecific supersensitivity was produced. Responses to all agonists tested were potentiated but no change in maximum responses were found. Though no receptor reserve was found in our experiments on denervated spleen, a number of workers report changes in pD_2 values of phenoxybenzamine after denervation (Trendelenburg, 1965; Karr, 1966), and this increased sensitivity to blockade by phenoxybenzamine could be the result of a change in conformation of the adrenergic receptors.

Phenoxybenzamine may bind at a site common to many types of receptors to produce blockade, thus accounting for the ability of phenoxybenzamine to antagonize responses to many classes of agonists; whether these sites are increased or altered by denervation has not yet been tested, those workers who reported changes in pD_2 values determined only values against noradrenaline. The design of our experiments to determine affinity must be commented on again, in that our results, while agreeing with predicted values, could obviously be misleading if tested on tissues where a change in affinity of phenoxybenzamine had occurred. Therefore before testing for possible changes in affinity in tissues where treatment has altered the normal sensitivity to agonists, determinations must be made of the sensitivity to phenoxybenzamine blockade. Innes (Personal Communication) first tested sensitivity to phenoxybenzamine and then

possible changes produced by cocaine in the affinity of sympathomimetic amines for alpha adrenergic receptors in cat spleen, and states that no changes could be detected.

Cocaine tested at concentrations from 10^{-6} to 10^{-4} g/ml had no further sensitizing effect on responses to agonists in strips from denervated spleen. This agrees with the results of other workers (Cannon, 1939; Innes and Kosterlitz, 1954; Kalsner and Nickerson, 1969), and has been used as one argument for supporting the uptake hypothesis which attributes potentiation by cocaine to block of uptake of amines into nerve cells, and this of course cannot occur if the nerves have already degenerated. However, this does not account for the unspecificity of the supersensitivity produced by cocaine or by denervation and an alternative explanation is that the changes in excitation-contraction which produce the supersensitivity are maximal after denervation and that cocaine therefore cannot produce any further changes in sensitivity. Cocaine, 3×10^{-5} g/ml, produces the same shift to the left of dose-response curves to agonists as does denervation, and a study of the effect of denervation on tissues where animals had already been fully sensitized by chronic treatment with cocaine, might shed some light on whether cocaine and denervation do have common mechanisms of action in production of supersensitivity.

COCAINE, RESERPINE AND DENERVATION ON CATECHOLAMINE CONTENT AND UPTAKE

The results obtained on strips of spleen do not support the hypothesis that cocaine generally causes supersensitivity by inhibiting the uptake of catecholamines. Cocaine potentiated both noradrenaline and isoprenaline but inhibited uptake of noradrenaline only. After

cocaine the dose-response curves for isoprenaline and noradrenaline were both shifted to the left without an increase in the maximum contraction. Our results with isoprenaline are in agreement with those of Seszkovszky and Tardos (1968) who showed potentiation by cocaine of a single selected dose of isoprenaline.

Uptake of noradrenaline and isoprenaline was tested on strips from cats treated with reserpine in order to deplete noradrenaline stores. Thus uptake of the catecholamines could be estimated without the interference of a large amount of noradrenaline in the control tissues. This procedure should not affect the validity of the test of the uptake hypothesis, since cocaine potentiated noradrenaline and isoprenaline in strips from cats treated with reserpine nearly as effectively as in strips from normal cats. The results of experiments in strips from spleens previously denervated show that the major uptake of isoprenaline and noradrenaline is in the adrenergic nerves. In addition, for the tests of uptake we selected loading concentrations of noradrenaline and isoprenaline and an exposure time similar to the concentrations and exposure time used in the tests of potentiation. We did not expect the spleen to take up substantial amounts of isoprenaline from the lower loading concentrations, for other tissues do not readily take up isoprenaline (Iversen, 1964). However, isoprenaline was taken up almost as well as noradrenaline. Cocaine had no effect on the uptake of isoprenaline but strikingly inhibited uptake of noradrenaline. Potentiation of isoprenaline cannot therefore be attributed to inhibition of uptake and another mechanism of potentiation must be sought. The potentiation of noradrenaline was greater than of isoprenaline, however; so it is possible that inhibition of uptake may

have an additional effect in the case of noradrenaline. Our results agree with the conclusions of various workers that inhibition of uptake cannot fully account for the development of supersensitivity (Maxwell, Wastilla and Eckhardt, 1966; Bevan and Verity, 1967; Kalsner and Nickerson, 1969; Varma and McCullough, 1969).

Uptake of catecholamines in the spleen has not yet been as thoroughly studied as has uptake in the rat heart. Rat heart has two uptake mechanisms, $Uptake_1$, responsible for uptake at low loading concentrations and $Uptake_2$, responsible for uptake at higher loading concentrations (Callingham and Burgen, 1966; Iversen, 1967). Isoprenaline is not taken up significantly by $Uptake_1$ but is taken up by $Uptake_2$, while cocaine inhibits $Uptake_1$ but not $Uptake_2$. Our results suggest that the uptake mechanisms in the cat spleen and the rat heart are different since the concentrations of isoprenaline we have used in the spleen overlap the effective concentrations for $Uptake_1$ and $Uptake_2$ and uptake is not inhibited by cocaine. On the other hand, uptake of noradrenaline over the whole range of concentrations studied is inhibited by cocaine.

GENERAL CONCLUSIONS AND SPECULATION

Studies on supersensitivity have now been carried out for more than a century and though the problem has stimulated a vast amount of research and many important findings have been reported we still do not know the mechanisms which produce changes in sensitivity of tissues to stimulating agents.

That most procedures which cause potentiation produce an unspecific type of supersensitivity, coupled with the fact that no changes in affinity can be detected, strongly suggests that supersensitivity is

produced by some fundamental change in the muscle cells beyond the level of the usual receptors for drugs. An obvious possibility is an altered membrane permeability and indeed this was suggested by Cannon (1939). Carrier and Shibata (1967) and Carrier et al. (1967) have demonstrated decreases in the calcium, sodium and potassium levels in vascular smooth muscle made supersensitive by pretreatment with reserpine and they attribute these results to an increase in permeability.

Studies on the electrical properties of smooth muscle have so far been confined to characterising the normal values of membrane potential and other such parameters. Reflections of changes in permeability could be profitably studied by modern techniques in electrophysiology. Lenman (1965) demonstrated a consistent and significant decrease in the resting membrane potential of denervated skeletal muscle in mice, and Levine (1966) showed that permeability of denervated skeletal muscle to sodium and potassium is altered. Further studies on electromechanical and pharmacomechanical coupling in normal and supersensitive tissues by studies of ion fluxes and membrane potentials should give us more detailed knowledge of excitation-contraction coupling in smooth muscle.

The suggestion by Fleckenstein and Bass (1953) that the normal transmitter action kept tissues in a subsensitive condition and that removal of the transmitter produced supersensitivity has received attention from time to time but there have been no proposals as to mechanisms of action or how to test the hypothesis. I suggest that the transmitter controls the synthesis of cell proteins and that after a procedure which abolishes or reduces the normal mediator content of a tissue, different proteins may be synthesized and this could explain the supersensitivity.

That small molecules can act as a repressor or derepressor has been established in bacterial cell systems, and it appears feasible that noradrenaline or acetylcholine could behave similarly in mammalian cells.

Studies on incorporation of labelled amino acids, use of protein synthesis inhibitors and analysis of amino acid sequences by modern biochemical techniques would test this hypothesis. It has been established that nervous control of tissue synthesis does take place, in that in some amphibia where limb regeneration can occur, denervation prevents regeneration (Sebowitz and Singer, 1970).

This hypothesis could explain denervation supersensitivity, where fourteen days is often required for production of maximal sensitivity changes, and even the supersensitivity due to reserpine, where at least twenty-four hours is required to obtain the potentiating effects of the drug. However, changes in permeability or some other mechanism of action must be looked for with such drugs as cocaine where almost immediate supersensitivity can be produced in vivo and in vitro.

SECTION V

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