STUDIES OF THE MECHANISMS TERMINATING THE ACTION OF THE

SYMPATHETIC NERVE MEDIATOR (NORADRENALINE)

A Thesis

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by

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TO MY WIFE

JENNY

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ABSTRACT

It was demonstrated that the gradual relaxation of contrac-
tions of vascular smooth muscle after the washout of stimulant drugs
in aqueous media in vitro, is related to the gradually decreasing con-
centration of "active" drug in the vicinity of the appropriate tissue
receptors. This was suggested by the observation that rates of relaxa-
tion from contractions produced by various α adrenergic stimulants
differed, and that the rate of relaxation could be increased by non-
competitive interference with drug-induced tone. Experiments with
enzyme inhibitors and the technique of receptor protection confirmed
that the relaxation of rabbit aortic strips could be correlated with a
gradually declining concentration of agonist in the biophase of the
tissue.

These results indicated that relaxation after a drug-induced
contraction could be used as a measure of the termination of drug action,
but to equate this with the effects of endogenous mechanisms of inactiva-
tion it was necessary to eliminate loss by diffusion into the surround-
ing medium. This was accomplished by developing a technique of oil
immersion, in which the aqueous medium in the muscle chambers was re-
placed by mineral oil after the tissue had reached equilibrium, or a
steady state, with the drug under study. It was demonstrated that the
oil per se exerted no pharmacological action, and that it did not inter-
fere with tissue function or the exchange of gases between the tissue
and its environment, as reflected in contractile performance. The
selective slowing by iproniazid of relaxation after contractions pro-
duced by dopamine, a substrate for monoamine oxidase (MAO), but not by

a nonsubstrate, Cobefrine, indicated that the technique is capable of assaying the activity of individual endogenous mechanisms when used in combination with specific inhibitors of possible inactivation pathways.

Experiments with the oil immersion technique demonstrated that the primary mechanism for the enzymatic inactivation of both noradrenaline and adrenaline in aortic tissue is O-methylation. Inhibition of catechol-O-methyl transferase (COMT) impaired the rate of inactivation of a low concentration of noradrenaline considerably less than it did that of the same concentration of adrenaline. Inhibition of monoamine oxidase (MAO) had a negligible effect on the inactivation of either, if COMT activity was unimpaired. Comparison of the increases in relaxation time due to various combinations of inhibitors of inactivation pathways indicated that COMT and MAO function as if they were arranged anatomically in series, with the latter an effective alternate mechanism for the inactivation of noradrenaline and somewhat less effective for adrenaline. Experiments with a 100-fold higher concentration of these catecholamines showed that the major endogenous pathways of inactivation were deamination and O-methylation for noradrenaline and adrenaline, respectively. It was suggested that the high concentrations of agonist swamped the organized, anatomically arranged system for their inactivation, and resulted in a more or less simultaneous presentation of amine to both enzymes, revealing their relative capacities for handling the two catecholamines.

Enzymatic processes were found to make a considerably more important contribution than binding and storage mechanisms as assessed on the basis of the effects of cocaine and methylphenidate, to the

inactivation of low concentrations of noradrenaline and adrenaline, and to account for almost all of the inactivation of high concentrations.

The roles of various endogenous mechanisms in terminating the action of sympathomimetics, as determined by the technique of oil immersion, were compared with those assigned by the traditional method based on potentiation of responses to agonists. It was found that the latter can be grossly inaccurate, both in detecting major endogenous inactivating mechanisms and in quantitating their contributions to the termination of action. This appears to be because of the complication introduced by diffusion into the surrounding medium. This factor may be of particular importance where the interaction of two or more endogenous mechanisms is involved, as in the "series" arrangement of COMT and MAO.

Other experiments demonstrated that the presence of potentiation per se cannot be equated with effects on inactivation, either qualitatively or quantitatively. In particular, most of the potentiation produced by cocaine appeared to be unrelated to blockade of inactivation by transport into nerves. Using cocaine and GD-131, a β-haloalkylamine congener of Dibenamine and phenoxybenzamine with little α adrenergic blocking activity, as potentiating agents, the following evidence for this dissociation was obtained.

1) Potentiation of responses to phenylephrine by cocaine was unaltered after a 60 minute exposure of reserpinized, iproniazid pretreated aortic strips to this agonist. In the absence of both intraneuronal storage and metabolism, net uptake of amine by nerves should

have been markedly reduced, with a concomitant reduction in the poten-
tiation produced by cocaine if this were a result of blockade of nerve
membrane transport of amine.

2) Cocaine and methylphenidate potentiated some responses to amines
(histamine and 5-hydroxytryptamine) which appear not to be taken up and
stored in adrenergic nerves. Methylphenidate was tested for and found
to have no effect on the disposition of histamine.

3) Cocaine still effectively potentiated responses of aortic
strips to noradrenaline after their neuronal elements had been allowed
to degenerate during prolonged periods in the cold and at 37°C.

4) Both cocaine and GD-131 effectively potentiated responses of
aortic strips to methoxamine, although studies by the oil immersion
technique confirmed the complete absence of endogenous mechanisms for
the inactivation of this sympathomimetic.

5) Both procaine and cocaine virtually eliminated the residual
inactivation of phenylephrine in iproniazid pretreated strips, but only
the latter potentiated responses to this amine. Cocaine still effect-
ively potentiated responses to phenylephrine in the presence of procaine.

6) The increased concentrations of active amine in the vicinity of
tissue receptors which could result from the delay in inactivation pro-
duced by cocaine or GD-131 were inadequate to account for and were
poorly correlated with the potentiation produced by these agents.

A detailed comparison of the two potentiating agents, cocaine
and GD-131 indicated that their major effect was exerted through a
common mechanism but that a small part of the effect of cocaine, and up
to nearly half of that of GD-131 involved independent mechanisms, related
to their effects on mechanisms of inactivation. Cocaine and GD-131

produced almost identical potentiation of responses to methoxamine, an effect entirely unrelated to inactivation. When added in sequence, cocaine produced a minor, but reproducible, potentiation of responses to noradrenaline in the presence of GD-131, whereas GD-131 caused a reduced, but still considerable, potentiation in the presence of cocaine. In addition, inhibition of COMT markedly decreased the potentiation of responses to tyramine in iproniazid pretreated aortic strips by GD-131, and appeared to decrease those in otherwise untreated noradrenaline contracted strips. Inhibition of COMT also somewhat decreased the potentiation produced by maximal doses of cocaine. GD-131 potentiated responses to noradrenaline significantly more than did the maximally effective concentration of cocaine.

The results obtained with strips from reserpinized animals did not support the hypothesis that decreased inactivation due to inhibition of storage mechanisms is responsible for the potentiation of responses to certain sympathomimetic amines by reserpine. In fact, reserpinized strips tended to inactivate both noradrenaline and phenylephrine more rapidly than did control preparations. Reserpinized preparations were found to have a decreased efficiency of storage and binding of noradrenaline, with a diversion of amine to metabolic inactivation, but the diversion was to COMT, rather than to MAO as is currently believed.

Both cocaine and GD-131 were found to alter the endogenous disposition of sympathomimetic amines, but the major mechanisms involved were different. The effect of cocaine on inactivation of amines appeared to be primarily due to blockade of binding and storage, with a

lesser action inhibiting access of amines to sites of metabolic in-
activation. Although not an enzyme inhibitor, the major effect of
GD-131 on amine inactivation was to "simulate" enzyme inhibition,
apparently by preventing access of amines to the enzymes. It blocked
access to sites of binding and storage only at the maximal exposure
short of producing α adrenergic receptor blockade. The major evidence
adduced to support these mechanisms of action were as follows:

1) Impairment of the inactivation of noradrenaline by moderate
exposure to GD-131 (1×10^{-5} for 10 to 15 min.) and by cocaine (1×10^{-4})
was approximately additive.

2) Moderate exposure of aortic strips to GD-131 reduced the rate
of inactivation of noradrenaline more than did inhibition of either
MAO or COMT.

3) Cocaine produced a further reduction in the rate of inactivation
of noradrenaline by aortic strips in which both COMT and MAO had been
inhibited, but moderate exposure to GD-131 did not, indicating that
the major action of the latter is on access of the amine to sites of
enzymatic degradation and not on transport to sites of binding and
storage.

4) GD-131 potentiated responses to tyramine, whereas cocaine
inhibited them. This provided further evidence that the major action
of GD-131 is not on nerve membrane transport of amine to storage sites.

5) Maximal exposure to GD-131 short of producing α adrenergic
blockade sometimes inhibited the inactivation of noradrenaline as much
as did combined treatment with iproniazid, tropolone and cocaine, which
indicates that this compound has also the ability to block the binding

and storage of amines, as does cocaine.

6) Cocaine appeared to have minor components of action comparable to the major properties of GD-131. It produced a smaller decrease in the inactivation of phenylephrine and adrenaline in aortic strips in which MAO and COMT, respectively, had been inhibited than it did in the controls, indicating some overlap with the major pathway of amine metabolism in each case.

Experiments performed with tyramine, bretylium and guanethidine indicated that these agents all cause release of catecholamines from endogenous stores in concentrations producing no more than minimal responses in Krebs medium. The amplitude of contraction of strips exposed to any one of these agents increased after oil immersion to reach a plateau, which was sustained for the duration of oil immersion. Phenoxybenzamine blocked this response and its magnitude and rate of rise were markedly reduced in strips from reserpinized animals. The barrier to diffusion produced by the oil made it possible to demonstrate otherwise undetectable amounts of endogenously released noradrenaline. This was demonstrated by the fact that reserpinized preparations treated with any one of the above agents and unreserpinized strips treated with iproniazid plus tropolone plus cocaine consistently responded with gradual contractions in oil, although no response was visible in Krebs medium. The response of unreserpinized strips treated with the 3 inhibitors was probably due to noradrenaline "spontaneously" released from nerve endings, which was trapped by the oil and protected against endogenous inactivation by the combination of inhibitors.

The suitability of the technique of oil immersion for studies

of endogenous mechanisms of inactivation of agents other than sympatho-mimetic amines was demonstrated in experiments showing that MAO plays an important role in the inactivation of 5-hydroxytryptamine and that diamine oxidase and an additional mechanism, perhaps N-methyl trans-ferase, are responsible for the endogenous inactivation of histamine.

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I. GENERAL INTRODUCTION

Adrenergic mechanisms have traditionally been investigated in
smooth muscle systems in vitro, the interactions of drugs with the tis-
sues being assessed in terms of contraction or tension development.
More recently, biochemical techniques have been used intensively to
gain insight into various aspects of drug-tissue interactions. The
involvement of various electrolytes in smooth muscle contraction (Bohr,
1964) and the role of phosphorylase in catecholamine-induced responses
(Sutherland and Rall, 1960) are examples of the varied directions these
studies have taken. The inactivation of sympathomimetic amines by var-
ious organs and tissues has also been studied biochemically, using iso-
topically-labelled amines of high activity and sophisticated chromato-
graphic and counting procedures (Axelrod, 1959a,b).

Except for work concerned specifically with the nature of
drug-receptor interactions, the dissipation of drug effects with time
has received little attention. In some in vivo studies attention has
been directed to the duration of the effects produced by sympathomimetic
agents, and occasionally even the rate of decline of the effect has been
recorded. For example, Cannon and Rosenblueth (1949) noted that cocaine
prolongs as well as enhances the contraction of the nictitating membrane
and the rise in blood pressure induced by noradrenaline or adrenaline.
Although the fact is not commonly appreciated, many alterations in the
physiological state of smooth muscle are reflected in changes in both
the duration and the magnitude of its responses to drugs. For example,
Elliot (1905) showed that responses of smooth muscle to an agonist such
as adrenaline are increased in both duration and magnitude after decen-
tralization. Unfortunately, in vivo studies involve many variables

which confound attempts to interpret the decay of the response to a drug
in terms of the mechanisms involved in its disposition or inactivation
in a particular organ system. These include alterations in nerve activ-
ity and regional blood flow, as well as shifting concentration gradients
of drug, all of which can markedly influence the duration of action.

Few attempts have been made to utilize the recovery process
after washout of a stimulant drug from the medium surrounding a smooth
muscle structure in vitro as a means of gaining insight into adrenergic
mechanisms. In the case of vascular smooth muscle, exemplified by the
popular spirally cut aortic strip preparation, a possible explanation
for this neglect is at hand. It has been widely assumed that the slow
relaxation which occurs after washout of a stimulant drug is determined
by the physical properties of structural elements of the arterial wall
rather than by the declining concentration of drug within the tissue
(Furchgott and Bhadrakom, 1953).

Contemporary theories of adrenergic mechanisms rest heavily
on postulates which could be investigated by studying the rate of decay
of the effects of sympathomimetic amines on smooth muscle if a suitable
in vitro system could be developed. Termination of a response to a sym-
pathetic mediator or other sympathomimetic amine is obviously a function
of changes in the effective concentration of the drug at its site of
action. The relationship between effective concentration and response
is also basic to current theories which regard the potentiation of re-
sponses of smooth muscle to noradrenaline and adrenaline by diverse
agents (e.g., cocaine, antihistamines, etc.) to be merely reflections
of increased concentrations and durations of amine in the environment

of adrenergic receptors.

Many studies of the influence of enzyme inhibitors and of potentiating drugs on amine inactivation mechanisms done by determining their effects on responses to a catecholamine or other sympathomimetic are prejudiced by the fact that the investigator is committed to the position that potentiation of a response to a drug and inhibition of an inactivation pathway for that drug are two sides of the same coin, i.e., that one cannot occur without the other. Consequently, the presence or absence of potentiation is considered to be a measure of the relative importance or unimportance of the inactivation mechanism obtunded. In practice, one group of workers usually demonstrates the effect on a possible inactivation pathway, while another documents the potentiation, often under very different conditions. However, some preparations can provide both types of data, e.g., the perfused spleen can give both an assayable venous output of noradrenaline and recordable volume and tension changes (Thoenen et al., 1964). Attempts have also been made to study on aortic tissue both the changes in noradrenaline content and the potentiation of responses to this mediator induced by an agent such as guanethidine (Maxwell, 1965b).

Some of the conclusions drawn on the assumption that the presence or absence of potentiation is a measure of the importance of a possible inactivation pathway have been far-reaching. One of the most effective arguments against the importance of monoamine oxidase (MAO) in the inactivation of noradrenaline was the failure of the effective, irreversible MAO inhibitor iproniazid to potentiate responses of smooth muscle structures to this amine. Conversely, the most telling arguments

for the relative importance of catechol-O-methyl transferase (COMT) and

of nerve uptake as inactivation mechanisms have been the potentiation

of responses to noradrenaline by pyrogallol and cocaine, respectively.

The work reported in this thesis had two major objectives.

1) To attempt to develop a system which could be used to study both the

contractile responses of isolated smooth muscle to various drugs and

the inactivation of the stimulant within the tissue. The most obvious

indicator of inactivation was relaxation after a drug-induced contrac-

tion, but its use for this purpose required demonstration that the

course of relaxation after washout of a stimulant drug is determined by

changes in its concentration in the vicinity of the appropriate tissue

receptors. In addition, in order to attribute the decrease in drug

concentration to tissue inactivation mechanisms, it was necessary to

eliminate the complication of loss by diffusion into the fluid environ-

ment of the tissue. 2) To use the preparation developed in an attempt

to evaluate some of the current theories of adrenergic mechanisms.

II.   GENERAL METHODS AND MATERIALS

a.   Preparations of Aortic Strips:

Rabbits of mixed breed and of either sex were obtained from local suppliers and kept in the departmental animal house at least a few days before use. Only animals weighing between 2.0 and 4.0 kg were used. They were killed by a blow on the back of the head and subsequently bled through severed throat blood vessels. The entire length of the descending thoracic aorta was quickly but gently excised and placed at once in a small beaker containing previously aerated (95% O_2 and 5% CO_2) modified Krebs-Henseleit (Krebs) solution at room temperature. Following transport to the laboratory the aorta was placed in a petri dish containing a sheet of filter paper moistened with the Krebs solution and adherent fat and connective tissue trimmed off as completely as possible. During this and subsequent manipulations the aorta was repeatedly dipped in Krebs solution to ensure that no dehydration occurred. The aorta was then held gently in the left hand and a long continuous spiral strip about 2.5 mm in width cut by rotating the aorta towards a fine scissors held in the right hand (Furchgott, 1960a). Segments about 2.3 cm in length were cut from this for mounting in individual muscle chambers. Eight strips could be routinely obtained from a single aorta. Each strip was attached by a loop of thread on one end to an aerating tube submerged in the chamber, and by a length of thread on the opposite end to an isotonic lever. The chambers were of the drainout type and contained Krebs solution constantly bubbled with a gas mixture containing 95% O_2 and 5% CO_2. The chambers were of approximately 10 ml working volume and were maintained at a temperature of 37 ± 0.5°C by circulating water from a thermostatically controlled bath through jackets surround-

ing the chambers. The placement of the circulating water bath was such
as to give a constant, slight vibration to the kymograph writing pens
to prevent sticking. The strips were placed under a tension of 2 grams
and contractions were recorded isotonically on a slowly moving (usually
1.4 mm/min.) kymograph paper with a 6.8 fold magnification. A 2 hour
period was allowed to elapse before the addition of any stimulant drug.
During this time the bath fluid was periodically changed.

b. Preparation of Drugs:

The following drugs were used:

DRUG	SOURCE
l-Noradrenaline* bitartrate	Calbiochem
l-Adrenaline* bitartrate	Sterling-Winthrop
Phenylephrine* hydrochloride	Sterling-Winthrop
5-Hydroxytryptamine* creatine sulphate	Calbiochem
Cobefrine* hydrochloride	Sterling-Winthrop
dl-Isoproterenol hydrochloride	Sterling-Winthrop
Methoxamine hydrochloride	Burroughs Wellcome
Dopamine hydrochloride	Mann Research
Tyramine hydrochloride	Calbiochem
Histamine diphosphate	Nutritional Biochemicals
Cocaine hydrochloride	British Drug Houses
Bretylium tosylate	Burroughs Wellcome
Phenoxybenzamine hydrochloride	Smith, Kline & French
Procaine hydrochloride	Ingram & Bell
Guanethidine sulphate	Ciba
Reserpine	Ciba

DRUG	SOURCE
Iproniazid phosphate	Hoffman-La Roche
Tropolone	Aldrich Chemical
Methylphenidate hydrochloride	Ciba
Semicarbazide hydrochloride	British Drug Houses
GD-131 hydrochloride	Givauden-Delawanna
Potassium chloride	Fisher
Sodium nitrite	Fisher
α-Methylphenethylhydrazine (J.B.-516, Catron)	Lakeside
Isoniazid phosphate	Hoffman-La Roche
Angiotensin amide	Ciba
Pyrogallol	British Drug Houses
α-Propyldopacetamide (H 22/54)	A.B. Hässle

* These drugs are referred to in terms of the weight of the base. Concentrations of all others are expressed as weight of the salt indicated, except potassium chloride which is referred to in terms of molarity.

Drug concentrations are expressed as final concentrations in the muscle bath in gm/ml (except KCl). Stock solutions of all drugs were made up in single distilled water which had been passed through an Illco-way ion exchange resin unless otherwise specified, and were kept at 8°C. HCl (0.01 N) was added to all stock solutions of catecholamines. All drugs were diluted to appropriate concentrations on the day of use with 0.9% NaCl, in the case of catecholamines containing 0.01 N HCl to prevent rapid oxidation. The dilutions were refrigerated when not in immediate use. Drugs were added to the muscle chambers with 0.1 and

0.2 ml blow-out pipettes, and were rapidly equilibrated with the bath fluid by the aeration stream. The total volume of drug solutions added was rarely over 0.5 ml and was usually 0.2 ml or less.

Phenoxybenzamine HCl was made up as a stock solution of 5 mg/ ml in propylene glycol, to which was added a few drops of 0.1 N HCl, and kept refrigerated at 8°C. Dilutions were made on the day of use in 0.9% NaCl containing 0.01 N HCl.

GD-131 HCl (N-cyclohexylemethyl-N-ethyl-β-chloroethylamine) was usually prepared on the day of use in a stock concentration of 1.0 mg/ml in distilled-demineralized water containing 0.1 N HCl. Dilutions were made in the same way as for phenoxybenzamine.

Angiotensin amide (Hypertensin) 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 8°C.

Reserpine solutions were made by dissolving the powder in 10% ascorbic acid to the appropriate concentration, and were stored at 8°C. Fresh solutions were made every few days.

c. Bathing Medium:

The muscle chambers were filled with a modified Krebs-Henseleit solution (Krebs) of the following composition:

Substance	g/l	mM/l
NaCl	6.74	115.3
KCl	0.35	4.6
$CaCl_2$	0.20	1.8
$MgSO_4$	0.14	1.1
KH_2PO_4	0.16	1.1

Substance	g/l	mM/l
NaHCO$_3$	1.86	22.1
Glucose	1.42	7.8
EDTA (disodium)*	0.01	

Total = about 300 milliosmolar

* Disodium EDTA (British Drug Houses, analar/grade) was routinely added to the Krebs reservoir to give a final concentration of 1×10^{-5} gm/ml. This was done to prevent any heavy metal contaminants from catalyzing oxidation of catecholamines.

Washout of a drug from a muscle chamber, unless otherwise specified, was accomplished by an initial three changes of the bath fluid, followed by one change every 5 minutes until relaxation was complete. When a strip was returned to Krebs solution after a period in oil, the same procedure was followed if any residual contraction persisted, otherwise the 3 initial washes were followed by only occasional changes of the bath fluid.

d. **Oils:**

The oils used were:

1. Peanut Oil - Planters Nut and Chocolate Company,
   Toronto, Canada.

2. Mineral Oil - Liquid Petrolatum, U.S.P. 180-190 cstks.,
   Ardee Laboratories, Winnipeg, Canada.

3. Silicone Oil - L-45 - Viscosity, 20 cstks.,
   Union Carbide, New York, N.Y.

Flasks containing the oil were kept at 37°C in a water bath and constantly bubbled with 95% O_2 and 5% CO_2. When it was desired to add oil to the muscle chamber, the Krebs solution was drained out and the oil rapidly layered into the chamber from above by means of a pipette

with a large tip opening. The flow of O_2 and CO_2 was maintained through-out the period during which the tissues were immersed in oil. Unless otherwise specified, the oil used was mineral oil.

e. Enzyme Inhibition:

Iproniazid. The general procedure to inhibit monoamine oxi-dase was to add 1 or 2×10^{-4} gm/ml (usually 2×10^{-4}) iproniazid to the muscle chambers for 30 minutes. The iproniazid was then washed out and an additional 30 minutes, with frequent washes, allowed to elapse before other drugs were added.

Tropolone. The general procedure to inhibit catechol-0-methyl transferase was to add 3×10^{-5} or 1×10^{-4} (usually 3×10^{-5}) tropolone to the muscle chambers. Thirty to 60 minutes later, with the tropolone still present, drug testing was either begun or resumed. The same con-centration of tropolone was maintained throughout all subsequent tests. Although the action of this compound can be terminated by washing out, tropolone treated strips were not used for subsequent studies unless specifically indicated.

Pyrogallol. Because this catechol-0-methyl transferase inhibi-tor is highly unstable in alkaline solution, its oxidation products turn-ing the bathing fluid and tissues a deep brown colour, a special proce-dure for its use was adopted. Pyrogallol (1×10^{-4}) was added to the muscle chambers and every 5 minutes the bath fluid was changed and the pyrogallol readded. After about 20 minutes exposure, the pyrogallol was washed out and 10 minutes later drug testing was either begun or resumed. Care was also taken to ensure that aeration was not excessive during the use of pyrogallol as rapid aeration hastened its oxidation.

f. Measurement of Relaxation:

The relaxation of aortic strips was recorded on a kymograph, usually at a drum speed of 1.4 mm/min. The residual contraction at any time could be measured to the nearest millimeter on the shellacked record by the use of two rules held at right angles to each other. The accuracy was such that measurements obtained independently by two investigators rarely differed. Percent relaxation was calculated as the decrease from the amplitude of contraction immediately before washing the stimulant drug from the chamber or oil immersion. This was not always the peak amplitude of contraction as a variable fade from the peak to a sustained plateau height often occurred. The aortic strips were usually exposed to a stimulant drug for 20 to 30 minutes before washout of the chamber or oil immersion to allow the contractions for all treatment conditions to reach a stable plateau.

Changes in the degree of relaxation at a given time and changes in contraction height were converted to equivalent concentrations of drug on the basis of standard dose-response curves for each agent determined on aortic strips not otherwise exposed to drugs (Fig. 1).

g. Statistics:

Mean values were compared by Student's "two-tailed" t test for unpaired data except where the t test for paired data (Goldstein, 1964) is specifically indicated. A probability of 5% or less was considered significant unless another criterion is specifically indicated. Means are reported $\pm$ their standard error.
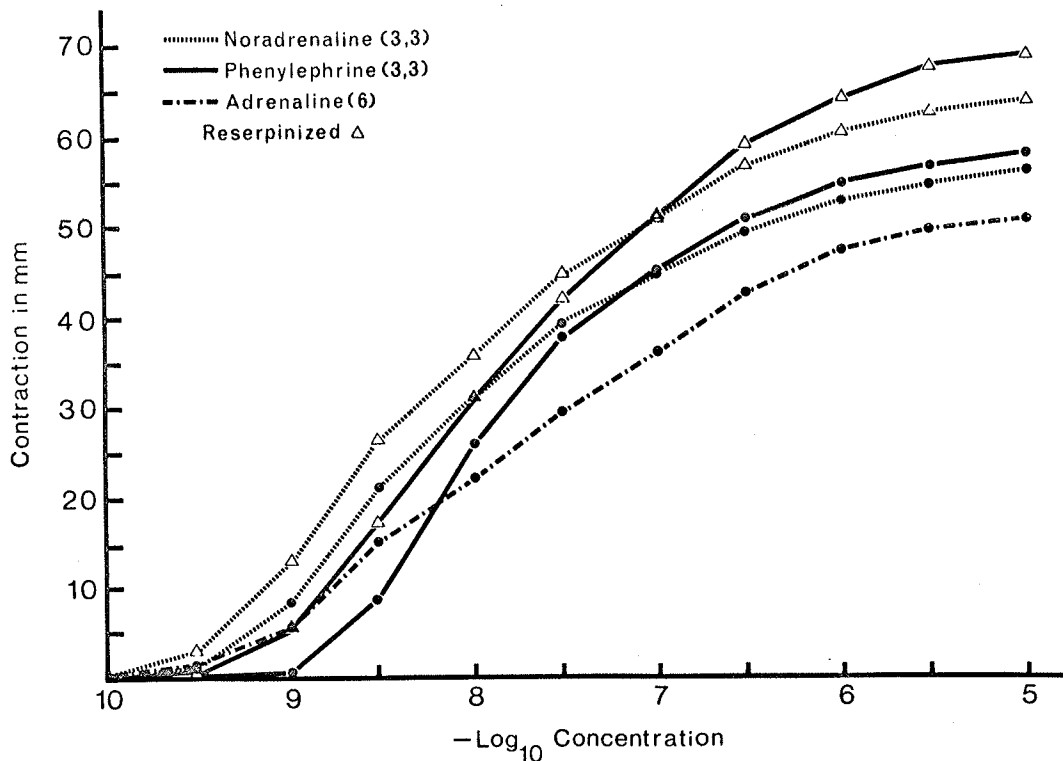
Fig. 1. Cumulative Log Dose-Response Curves for Contraction of Aortic Strips by Phenylephrine, Noradrenaline and Adrenaline.

Figures in parentheses indicate the number of complete experiments on which each curve is based.

III. DEVELOPMENT OF A METHOD FOR THE
STUDY OF ENDOGENOUS ROUTES OF DRUG INACTIVATION

A. RELATION OF RELAXATION IN AQUEOUS MEDIUM TO
PERSISTENCE OF DRUG IN AORTIC STRIPS

Spirally cut strips of blood vessels have been used extensively

as an in vitro test system for studies on the pharmacology of smooth mus-

cle. The spirally cut rabbit aorta preparation was included as part of

a classroom exercise in Sherrington's (1919) Laboratory Manual in Mam-

malian Physiology, and a similar preparation from the pig carotid artery

was described at length by Lewis and Koessler (1927). Furchgott and

Bhadrakom (1953) and Furchgott (1955) studied the responses of spiral

strips of rabbit aorta to a number of drugs and stressed the value of

this preparation in the quantitative investigations of smooth muscle

stimulants. Its advantages include a low or absent inherent (basal)

tone and consistent responses to many different drugs. The preparation

is very sensitive to noradrenaline, usually responding with a small con-

traction to a concentration of 1 X 10^{-9} of the base. The thinness of

the strip (about 0.4 mm) probably assures adequate oxygenation of and

drug access to all component cells. The aorta is innervated only by

sympathetic nerves, which eliminates possible complications due to the

presence of cholinergic ganglia and acetylcholine stores. The muscle

layer (media) makes up almost half of the vessel wall in the rabbit thor-

acic aorta (Elchlepp and Furchgott, 1955), and within it, the predomin-

ant orientation of cells is circular. There is little or no longitudin-

ally oriented muscle.

The relaxation of an aortic strip from a contraction of near

maximal amplitude produced by any one of a variety of agonists extends

over many minutes after washout of the drug (e.g., Kohli, 1965). After

contractions produced by phenylephrine (1 X 10^{-5}) and Cobefrine (1 X 10^{-5}),

about 35 and 60 minutes, respectively, are required for 90% relaxation

(Fig. 3). It has been generally assumed that this slow relaxation is

related to the time required for the lengthening of some component of

the aortic strip not involved in active contraction, and that the pro-

cess is unrelated, except in its initiation, to the concentration of

drug within the tissue. The origin of this assumption is not clear, but

it is implicit in even some of the earliest work on arterial smooth mus-

cle preparations (Lewis and Koessler, 1927). The slow relaxation limits

the frequency of drug testing. However, many workers consider that this

limitation can be overcome by the expedient of temporarily imposing ad-

ditional tension on the partially relaxed strip to hasten its return to

baseline and, thus, the resumption of drug testing (Furchgott et al.,

1963; Maengwyn-Davies, 1965).

Furchgott and Bhadrakom (1953) interpreted the initial phase

of relaxation to be a reflection of drug removal from the biophase (the

immediate environment of the tissue receptors) and the later phases to be

limited by the mechanical properties of some unidentified structural com-

ponent of the arterial wall. They did not clearly indicate the point at

which the rate limiting process changed, but only the initiation of re-

laxation was definitely attributed to a decreasing effective drug con-

centration. In the absence of any direct evidence that passive mechani-

cal events dominate a major part of the relaxation process, it appeared

possible that the time course of the greater part of the relaxation after

washout of a stimulant drug is determined by the rate of its removal from

the biophase. If this could be proved, relaxation could be used as an

index of drug inactivation in studies of the mechanisms by which vascu-

lar smooth muscle terminates the actions of sympathomimetic amines and other stimulant drugs.

1. RESULTS

a. <u>Comparison of Relaxation Rates after Various α Adrenergic Stimulants:</u>

Contractions of moderate and about equal magnitude were produced by noradrenaline, phenylephrine and isoproterenol in strips cut from the same rabbit aorta. At the concentrations used these sympathomimetic amines all produce contractions through the α adrenergic receptors (Furchgott, 1955). It was noted, however, that the rates of relaxation after washout of the different agonists were dissimilar. This is illustrated in figure 1a, which shows typical relaxation traces for contractions induced by noradrenaline and isoproterenol; relaxation occurred at an intermediate rate following phenylephrine. Passive restretching of noncontractile elements should not differ following essentially equal contractions due to different agonists. In addition, the variations in rate of relaxation could not be attributed to differences in the pathway activated, the only apparent explanation was in differences in the rates at which the different agonists were removed from the environment of the receptors.

b. <u>Effect of NaNO$_2$ on Relaxation Rate:</u>

An aortic strip was contracted by noradrenaline (1×10^{-5}) and the rate of relaxation after washout of the agonist recorded. After recovery to basal tone, a second contraction was produced by the same concentration of noradrenaline, and midway in the relaxation of the strip,
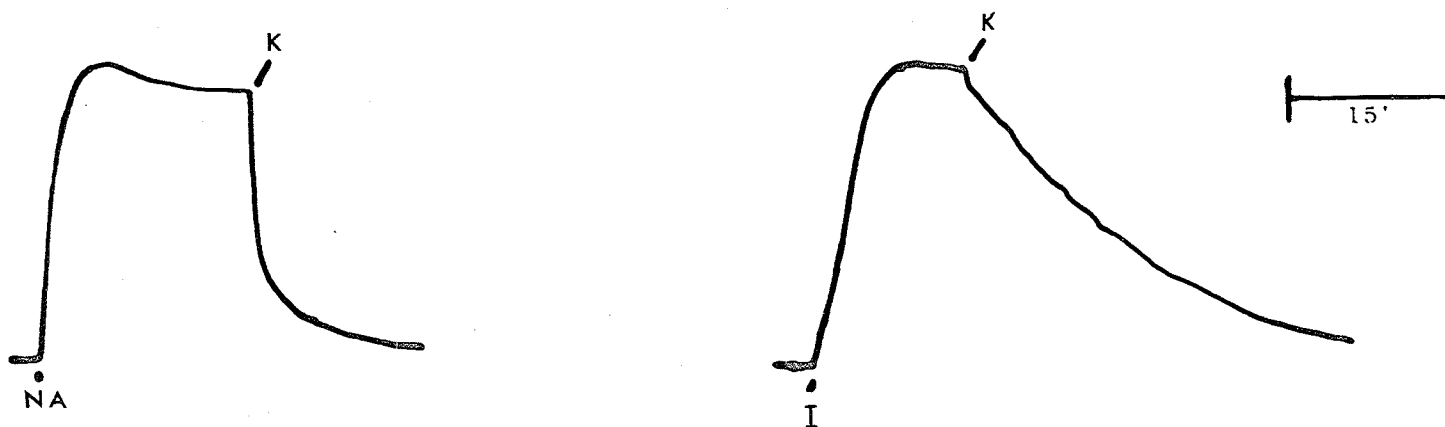
Fig. 1A. Relaxation of Aortic Strips after Contraction by Different α Adrenergic Stimulants.

Strips from the same aorta contracted with noradrenaline (NA) (1×10^{-8}) and isoproterenol (I) (1×10^{-5}). Drug washed out (K) as described in Methods.

$NaNO_2$ (1×10^{-3}) was added to the muscle chamber and readded after each wash. The records shown in figure 2 demonstrate that $NaNO_2$ markedly accelerated the return of the partially relaxed muscle to the baseline. The time to relax from 40 to 90% was decreased from 44 to 21 minutes by the $NaNO_2$. Figure 2 also shows the response of another strip from the same aorta which was treated with $NaNO_2$ after its first exposure to noradrenaline. A similar increase in the rate of relaxation was produced. $NaNO_2$ is believed to decrease smooth muscle tone through a noncompetitive action on some link in the contractile mechanism. $NaNO_2$ had no effect on uncontracted aortic strips, since tone is negligible in these preparations, and the records reproduced in figure 2 show that noradrenaline contracted strips do not relax below their previous baselines in the presence of $NaNO_2$.

c. Effect of Enzyme Inhibition on Relaxation Rate:

As a further test of the contribution of the presence of stimulant drug in the biophase to the course of relaxation, an attempt was made to alter the relaxation rate by inhibiting enzymes which might be involved in the disposition of the agonist. The length of time required for relaxation to be completed suggested that there would be ample opportunity for endogenous mechanisms as well as diffusion out of the tissue to contribute to termination of the stimulant action. Two sympathomimetic amines were chosen for this study: phenylephrine, because it has been shown to be a good substrate for monoamine oxidase (MAO), and Cobefrine, because the α-methyl substituent insures that it is not a substrate (Blaschko et al., 1937b; Blaschko, 1952). The presence of MAO in aortic smooth muscle cells was demonstrated by Koelle and Valk (1954).
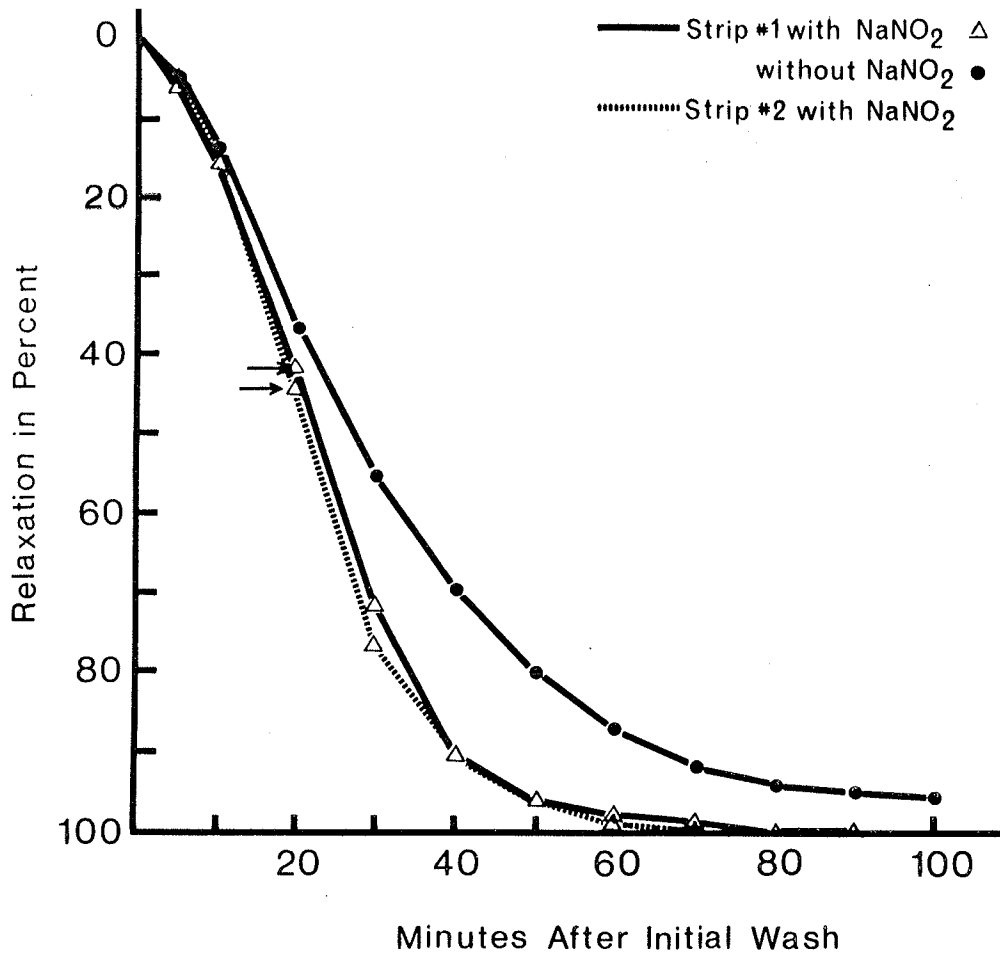
Fig. 2. Effect of NaNO$_2$ on the Rate of Relaxation of Aortic Strips after Washout of Noradrenaline

Strip #1 was contracted twice by noradrenaline (1 X 10^{-5}) for 30 minutes, and near the midpoint of relaxation from the second con-traction (20 min. after the initial wash, arrow) NaNO$_2$ (1 X 10^{-3}) was added and readded after each subsequent wash. Strip #2 was treated with NaNO$_2$ in the same way after its first exposure to noradrenaline (1 X 10^{-5} for 30 min.).

Aortic strips were pretreated with iproniazid as described in Methods, to inhibit MAO, and then near-maximal or maximal contractions were produced with phenylephrine (1×10^{-5} or 1×10^{-4}). The rates of relaxation of these strips after washout of the agonist were compared with those of strips treated identically except for the iproniazid pretreatment. In some experiments the animals from which strips were obtained had been pretreated with reserpine, but this had no effect on the rate of relaxation. As shown in Table I, the time required for relaxation from 60 to 90% after contractions produced by phenylephrine (1×10^{-4}) was signficantly increased by iproniazid pretreatment, from 20.4 ± 1.3 to 32.7 ± 3.6 minutes, and that for contractions produced by phenylephrine (1×10^{-5}) from 19.3 ± 1.7 to 26.2 ± 2.1 minutes. Iproniazid pretreatment increased the time for half relaxation of phenylephrine (1×10^{-5}) and (1×10^{-4}) contracted strips from 11 to 35 minutes and from 22 to 61 minutes, respectively (Fig. 3).

The animals from which aortic strips were obtained for the study of relaxation after contractions produced by Cobefrine (1×10^{-4}) were pretreated with reserpine (0.5 mg/kg intramuscularly, daily for two days) to eliminate any contribution of released noradrenaline to the response, since noradrenaline is substrate for MAO. The time required for relaxation from 60 to 90% was 27.5 ± 3.5 minutes in 2 control strips and was not significantly different for 2 iproniazid pretreated strips (29.5 ± 1.0 minutes); the time for half relaxation was similarly unaltered (Fig. 3). The failure of iproniazid to affect the relaxation rate of Cobefrine contracted strips demonstrated that inhibition of MAO was probably responsible for the observed effect on the rate of relaxation of

TABLE I

EFFECT OF IPRONIAZID (IPN) PRETREATMENT ON RATE OF RELAXATION
OF PHENYLEPHRINE CONTRACTED AORTIC STRIPS

Phenyl-ephrine Conc.	Time to Relax 60 to 90% (min.)		Contraction Height (mm)	
	Without IPN	With IPN	Without IPN	With IPN
1×10^{-4}	19	--	58	--
	17	27	57	62
	24	41	62	58
	18	23	69	77
	23	43	81	73
	25	37	80	72
	17	25	54	51
	$\bar{x} = 20.4 \pm 1.3$	$\bar{x} = 32.7 \pm 3.6$	$\bar{x} = 66$	$\bar{x} = 66$
	$P < 0.01 > 0.001$			
1×10^{-5}	16	29	54	58
	17	22	68	53
	21	33	77	59
	23	23	61	66
	--	24	--	51
	$\bar{x} = 19.3 \pm 1.7$	$\bar{x}\ 26.2 \pm 2.1$	$\bar{x} = 65$	$\bar{x} = 57$
	$P < 0.05 > 0.02$			

Values appearing on the same line are observations on strips from the
same aorta.
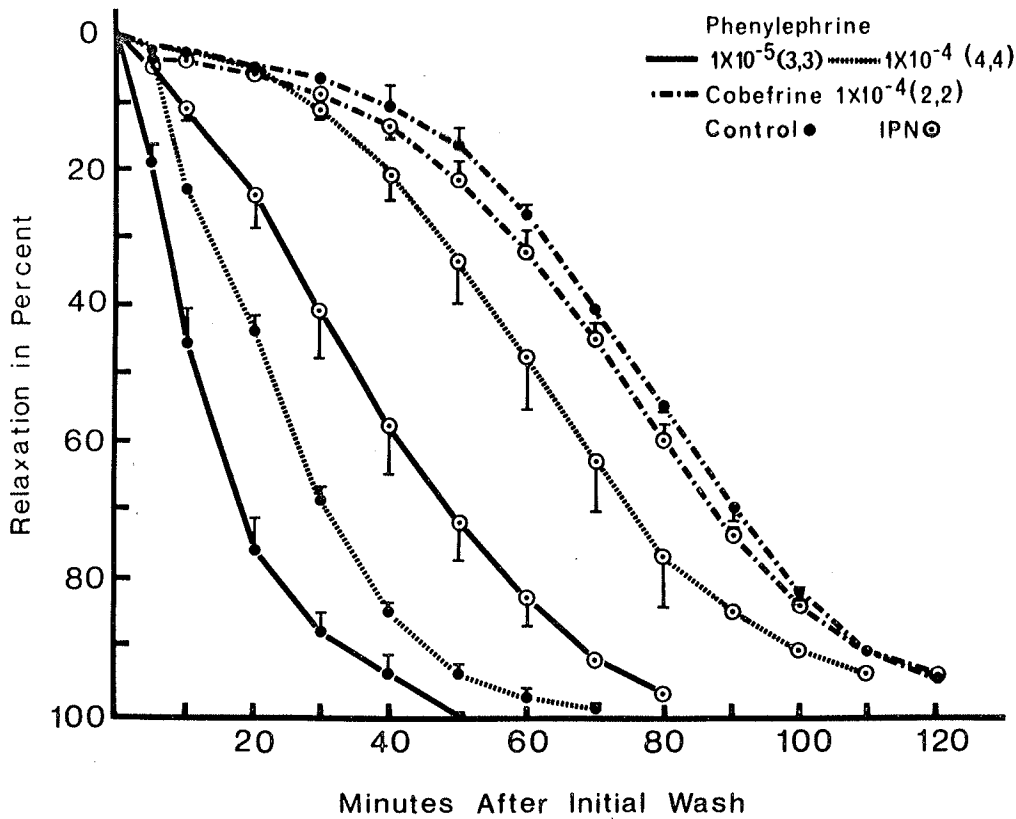
Fig. 3. Effects of Iproniazid on the Relaxation of Aortic Strips Contracted by Phenylephrine and Cobefrine.

The aortic strips used in experiments with Cobefrine were from reserpinized animals. Figures in parentheses indicate the number of experiments represented by each curve. Bars indicate the standard errors of means.

strips contracted with phenylephrine. The effect of enzyme inhibition on the final 10% of the relaxation process was not studied because the rate is very slow and differences would have been difficult to establish.

d. Protection of α Adrenergic Receptors during Relaxation:

The technique of "receptor protection" has been widely employed to provide evidence for the presence of an agonist or competitive antagonist on the α adrenergic receptors (Furchgott, 1954). The ability of the drug under study to protect the receptors from blockade during a short exposure to a β-haloalkylamine adrenergic blocking agent is considered to be a measure of its occupancy of the receptors. The degree of blockade is assessed with a standard α receptor agonist after both the "protecting" agent and unreacted β-haloalkylamine are washed out of the tissue. The concentration of β-haloalkylamine and the duration of exposure to it must be such that it will not occupy competitively all or most of the α adrenergic receptors even in the presence of another agent which can combine with them.

The receptor protection technique was used to determine if α receptors are still occupied by agonist at various stages during the gradual return of an aortic strip to baseline tone after washout of a sympathomimetic. Phenoxybenzamine (POB) was the β-haloalkylamine used in this study, and preliminary testing showed that exposure of quiescent aortic strips to a concentration of 1×10^{-8} for 10 minutes caused a marked shift to the right and depression of the maximum of the noradrenaline dose-response curve (Fig. 4b). The shift in the dose-response curve was as great as 1000-fold in some experiments. The same treatment of a strip while contracted with noradrenaline (1×10^{-5}) usually resulted in

Fig. 4. Protection of α Adrenergic Receptors against Phenoxybenzamine
Blockade after Washout of Noradrenaline.

Test responses of 3 strips to cumulative noradrenaline
(NA) (1 and 3 X 10^{-9}) are shown on the left. Strip a was then contracted
with noradrenaline (1 X 10^{-5}) for 15 minutes, followed by phenoxybenz-
amine (POB) (1 X 10^{-8}), and was washed (K) 10 minutes later. Strip b
was exposed to phenoxybenzamine (1 X 10^{-8}) alone for 10 minutes and then
washed. Strip c was contracted with noradrenaline (1 X 10^{-5}) for 25
minutes, followed by washout. Phenoxybenzamine (1 X 10^{-8}) was added 12
minutes later and washed out after 10 minutes. Relaxation was re-
corded on a standing drum except for brief marking periods. Responses
to cumulative additions of noradrenaline determined on each strip about
one hour after the end of exposures to phenoxybenzamine are shown on
the right. Noradrenaline concentrations (dots) are in sequence:
1 and 3 X 10^{-9}, 1 X 10^{-8}, 1 X 10^{-7}, 1 and 3 X 10^{-6}. All 3 strips were
from the same aorta.
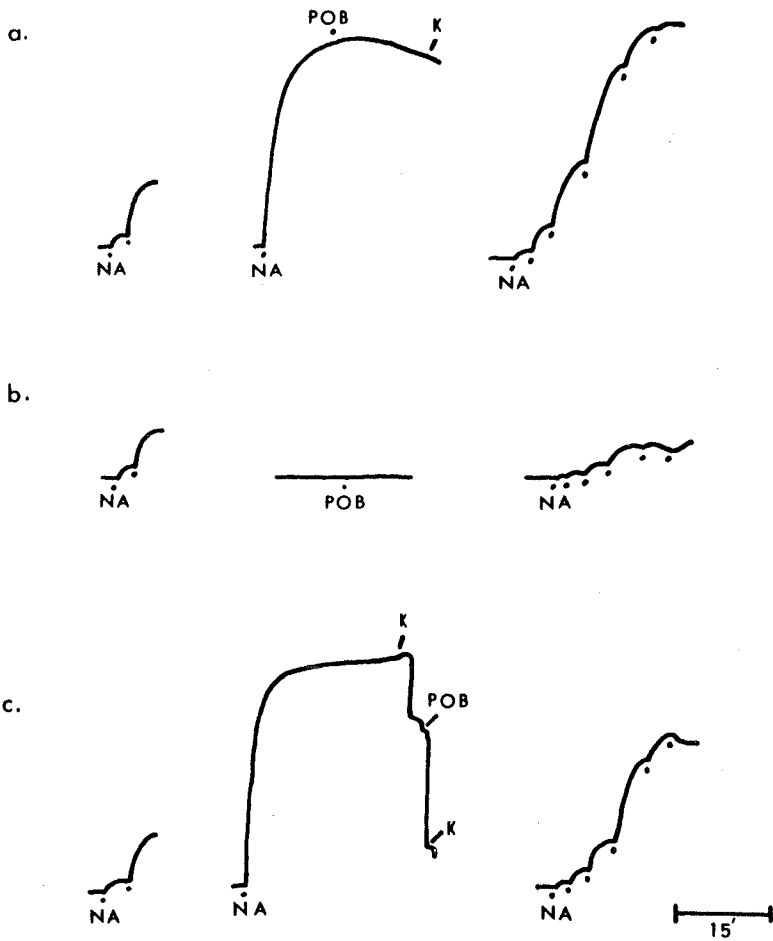
little or no blockade of the α adrenergic receptors (Fig. 4a).

Figure 4c shows the dose-response curve of a strip from the same aorta as the other two, which was treated with POB for 10 minutes, starting 12 minutes after washout of the noradrenaline. Partial protection of the receptors in this strip is indicated by a lesser increase in the threshold concentration and by a considerably smaller reduction in maximal height of contraction than was produced by POB in the strip not exposed to noradrenaline. During the 10 minutes of exposure to POB this strip relaxed considerably, indicating successful competition of the POB with noradrenaline for some of the receptors. Although the strip had relaxed about 80% at the time the POB was washed out, the degree of receptor protection was considerable.

The results of an experiment which clearly demonstrated partial protection of the receptors up to 40 minutes after washout of noradrenaline (1×10^{-5}) are shown in figure 5. Five strips were cut from the same aorta and test responses to low concentrations of noradrenaline obtained to establish matching sensitivities. Strips #1 to #4 were exposed to noradrenaline (1×10^{-5}) for a total of 25 minutes and all drug then washed out of the chambers. Strip #1 was exposed to POB (1×10^{-8}) for 10 minutes, starting 15 minutes after the administration of the noradrenaline. Strips #2, #3 and #4 were also exposed to POB for 10 minutes, starting 5, 15 and 30 minutes after washout of the noradrenaline, respectively. Strip #5 was not exposed to noradrenaline after the initial tests of sensitivity, but was exposed to POB for 10 minutes. Subsequently determined cummulative dose-response curves to noradrenaline clearly showed partial protection against the POB blockade in strips #2 to #4.
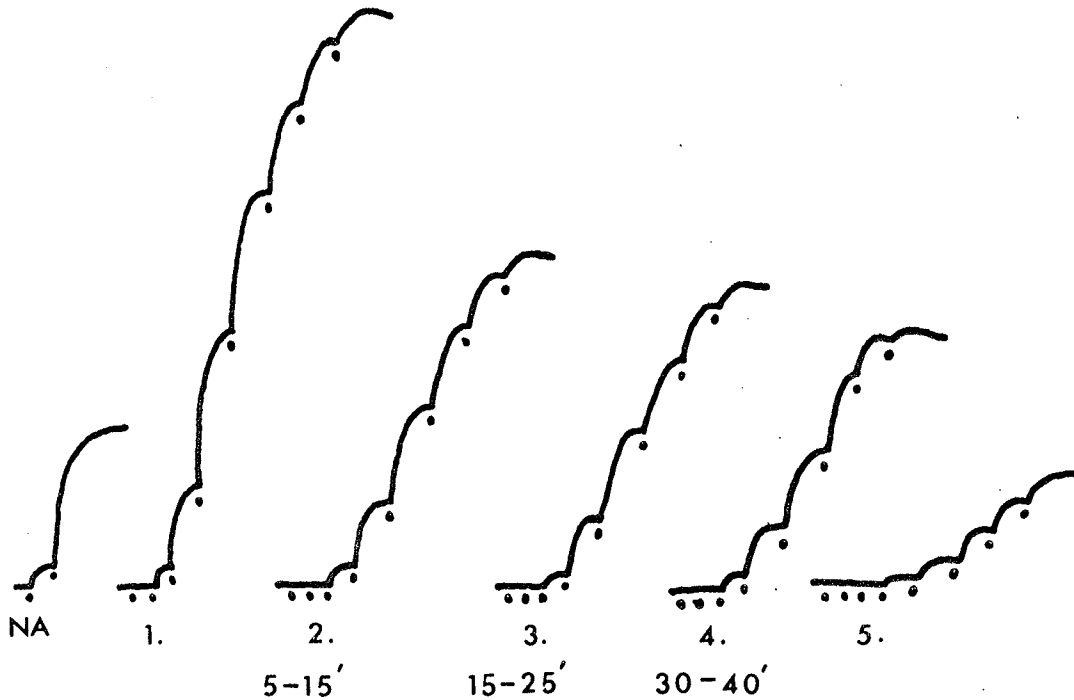
Fig. 5. Protection of α Adrenergic Receptors against Phenoxybenzamine Blockade at Various Times after Washout of Noradrenaline

Record on extreme left (same strip as #1) is representative of responses of all 5 strips from a single aorta to test concentrations of noradrenaline (1 and 3 X 10^{-9}). All strips were exposed to phenoxybenz-amine (1 X 10^{-8}) for 10 minutes, #1 in the presence of noradrenaline (1 X 10^{-5}), strips #2 to #4 at the indicated times after washout of nor-adrenaline (1 X 10^{-5}), and strip #5 in the absence of noradrenaline. The responses to cumulative concentrations of noradrenaline were obtained simultaneously for all 5 strips over one hour after end of exposure to phenoxybenzamine. Noradrenaline concentrations (dots) are in sequence: 1 and 3 X 10^{-9}, 1 and 3 X 10^{-8}, 1 and 3 X 10^{-7}, 1 and 3 X 10^{-6}.

Protection of α receptors was studied in a total of 14 strips from 9 aortas exposed to POB beginning 5 to 30 minutes after washout of noradrenaline (1×10^{-5}). Partial protection of the α adrenergic receptors, which decreased progressively with time, was demonstrated in all tests. For example, 3 sets of 3 strips each were prepared from the same aortas. One set was exposed to POB without noradrenaline, and the other 2 were exposed beginning 15 and 30 minutes after washout of the noradrenaline (1×10^{-5}). The subsequently determined mean peak contractions for the 3 groups were 21.7, 34.7 and 41.3 mm, respectively. The degree of relaxation at the time of washout of POB was not routinely recorded in these experiments, but in most cases it was well over 50% (Fig. 4c).

The degree of protection against blockade by POB added 15 minutes after washout of noradrenaline (1×10^{-5}) was compared in control strips and strips pretreated with iproniazid as described in Methods. The iproniazid pretreated strips relaxed considerably less than did the controls in the 15 minutes before POB was added, and concomitantly, were protected more effectively against the α adrenergic blockade. A comparison of 4 control strips and 4 pretreated with iproniazid, cut from the same aortas, showed the latter to have consistently higher maximal responses and 3 of them to have lower thresholds for responses to noradrenaline after the exposure to POB (Fig. 6 and Table II).

Iproniazid pretreatment alone did not alter the blockade produced by POB, and had no apparent effect on the noradrenaline dose-response curve. Contractions produced by noradrenaline (3×10^{-9}), given either in one dose or cumulatively, 3×10^{-10}, 1×10^{-9} and 3×10^{-9}, were measured in 8 strips before and after treatment with iproniazid,
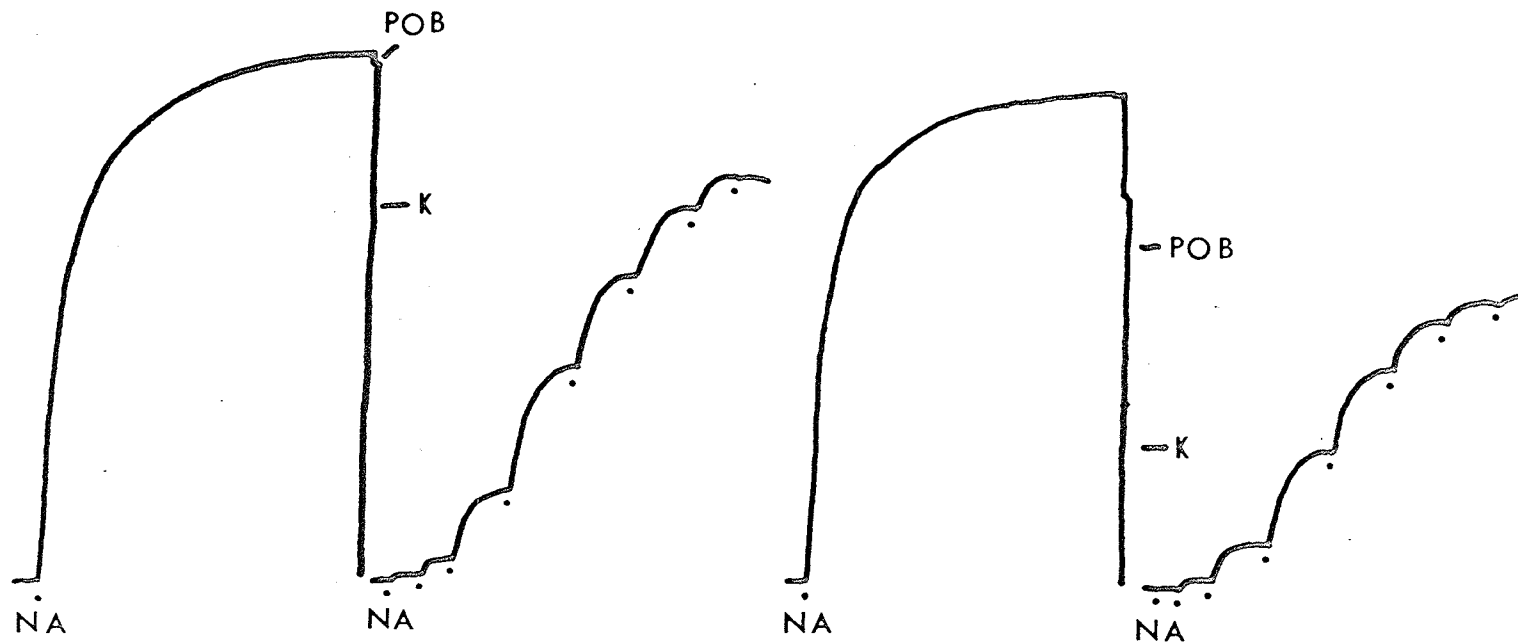
Fig. 6.   Effect of Iproniazid on Protection of α Adrenergic Receptors against Phenoxybenzamine Blockade after Washout of Noradrenaline.

Responses of a strip pretreated with iproniazid (left) and of a control strip from the same aorta. Both were contracted with noradrenaline (NA) (1 X 10^{-5}) and exposed to phenoxybenzamine (1 X 10^{-8}) for 10 minutes (POB to K), beginning 15 minutes after washout of the noradrenaline. Relaxations after washout of noradrenaline were recorded on a standing drum. Responses to cumulative additions (dots) of noradrenaline (1 X 10^{-9} to 3 X 10^{-6} as in Fig. 5) were obtained about one hour after end of exposure to phenoxybenzamine.

TABLE II

EFFECT OF IPRONIAZID (IPN) ON PROTECTION AGAINST PHENOXYBENZAMINE

(POB) BLOCKADE AFTER WASHOUT OF NORADRENALINE

	Responses to Noradrenaline (1×10^{-5}) (mm)		Maximal Contraction after POB (% of Control)		Threshold after POB $(X\ 10^{-9})$	
	Without IPN	With IPN	Without IPN	With IPN	Without IPN	With IPN
1.	69	74	59.4	75.7	3	1
2.	49	52	57.1	63.5	3	3
3.	86	75	48.8	70.7	3	1
4.	35	33	62.9	72.7	3	1
	$\bar{x} = 59.8$	$\bar{x} = 58.5$	$\bar{x} = 57.1$	$\bar{x} = 70.7$		

Values appearing on the same line are observations on strips from the same aorta matched for initial sensitivity to noradrenaline. Cumulative noradrenaline additions and procedures were as described for figure 6. Threshold concentration is that giving first detectable response.

and in 8 strips at the same time interval, but without the intervening
inhibition of MAO. The treatment with iproniazid had no effect on the
heights of the contractions produced by either 1×10^{-9} or 3×10^{-9} nor-
adrenaline (Table III).

e. Ancillary Studies:

Studies performed with lower concentrations of phenylephrine
and with a wide range of concentrations of noradrenaline indicated a
major role for endogenous mechanisms in terminating the in vitro action
of these compounds after the chambers are washed free of drug (Table IV).
Control, noradrenaline (1×10^{-5}) contracted strips relaxed $42.6 \pm 8.6\%$
in 15 minutes, whereas iproniazid pretreated strips relaxed only $3.9 \pm$
1.4% in the same interval of time. After noradrenaline (1×10^{-6}), the
mean percent relaxation 5 minutes after washout was decreased from 52.8
in control strips to 9.1 in catechol-O-methyl transferase (COMT) plus
MAO inhibited strips whereas inhibition of only one of these enzymes re-
sulted in an intermediate impairment of the rate of relaxation. After
iproniazid pretreatment a strip contracted with noradrenaline (1×10^{-7})
required 4 minutes for 50% relaxation and the same strip after pretreat-
ment with iproniazid plus tropolone required 13 minutes to relax 50%.

The results obtained with moderate concentrations of phenyl-
ephrine (Table IV) indicated that the effect of iproniazid decreased with
decreasing agonist concentration, such that the time for half relaxation
of strips contracted by phenylephrine (1×10^{-7}) was increased only 50%.

TABLE III

EFFECT OF IPRONIAZID (IPN) ON CONTRACTION OF AORTIC STRIPS BY NORADRENALINE

Noradrenaline Conc.	First Contraction (mm)	Interval Treatment	Second Contraction (mm)	Change in Response
1×10^{-9}	10.0 (5 - 17)	--	4.0 (2 - 8)	- 53.2 ± 13.2% (4)
	5.8 (4 - 8)	IPN	4.0 (2 - 10)	- 37.9 ± 21.3% (4)
				N.S.
3×10^{-9}	22.8 (18 - 32)	--	22.8 (12 - 33)	- 0.41 ± 7.0% (8)
	21.1 (13 - 31)	IPN	22.4 (12 - 37)	+ 3.6 ± 4.3% (8)
				N.S.

Figures in parenthesis on the extreme right indicate number of strips in each group, those to the right of contraction amplitudes, the range of values. Changes in response were determined on the basis of intraindividual comparison.

TABLE IV

EFFECT OF ENZYME INHIBITION ON RATES OF RELAXATION OF PHENYL-
EPHRINE AND NORADRENALINE CONTRACTED AORTIC STRIPS

Noradrenaline

Agonist Conc.	Control		Iproniazid		Pyrogallol		Combined Inhibitors*	
	Min.	%	Min.	%	Min.	%	Min.	%
1×10^{-7}			4	50			5	16
,			5	59			10	34
			10	76			13	50
1×10^{-6} (2)	5	52.8	5	43.9	5	35.8	5	9.1
1×10^{-5} (4)	15	42.6	15	3.9				
		± 8.6		± 1.4				

Phenylephrine - 50% Relaxation Time (min.)

Agonist Conc.	Control	Iproniazid
1×10^{-7}	4	6
3×10^{-7}	6	11
1×10^{-6}	6	12
1×10^{-5} (3)	11	35
1×10^{-4} (4)	22	61

*Iproniazid plus pyrogallol except for strips contracted by noradrenaline (1×10^{-7}) which were exposed to tropolone instead of pyrogallol.

Relaxation is given as % at the indicated times after wash-
out of agonist. Values appearing on the same line (except
means) are for strips from the same aorta. Figures in par-
enthesis indicate number of strips included in means.

2. DISCUSSION

The sum of the evidence presented in this section strongly favors the conclusion that the course of relaxation of an aortic strip after washout of a sympathomimetic amine is determined by the gradually declining level of agonist in the environment of the α adrenergic receptors (biophase). The long time course of recovery to basal tone suggests that the agonists penetrate some diffusion barrier which limits their passage back into the bathing medium. The relationship of relaxation to residual drug concentration was confirmed by the finding that following contractions induced by phenylephrine or noradrenaline enzymatic inactivation of the amine plays an important role in determining the rate of relaxation.

A scheme for visualizing the parameters involved in the in vitro termination of drug action can be constructed on the model used by Furchgott (1955) in discussing drug potentiation.

Aqueous Phase   Biophase

$$D_a \xrightleftharpoons[k_b]{k_a} D_b + R \xrightleftharpoons[k_2]{k_1} RD \longrightarrow$$

$$D_b \xrightarrow{k_1'} \quad \xrightarrow{k_2'} \quad \xrightarrow{k_n'}$$
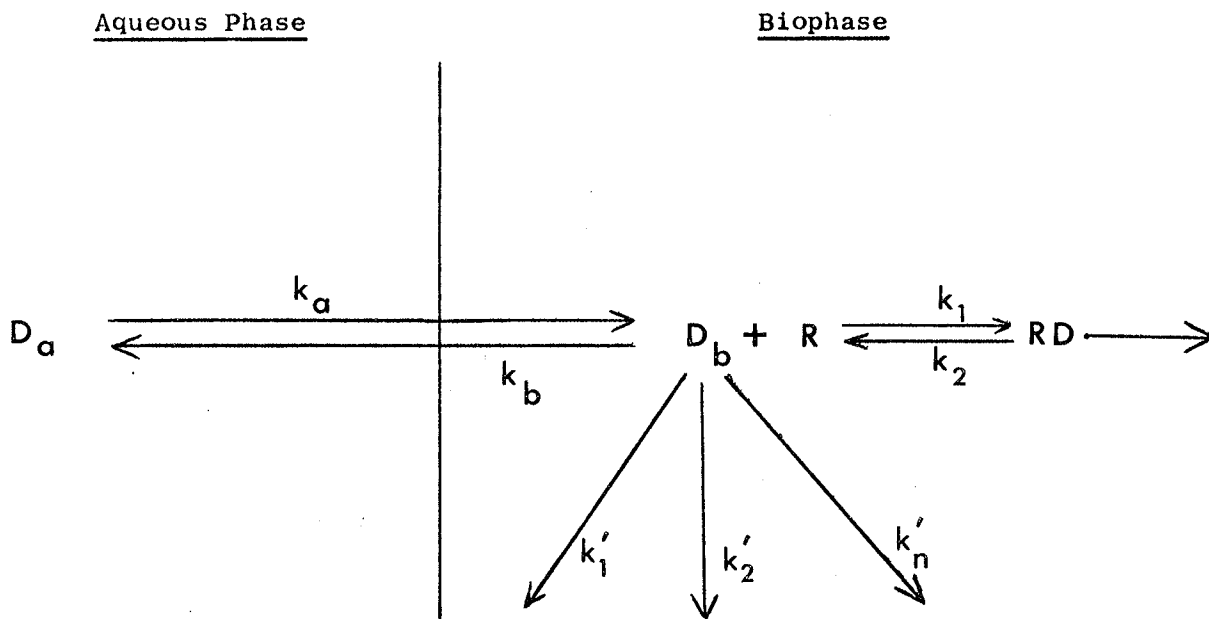
D_a and D_b represent the agonist (e.g., phenylephrine) in the external aqueous phase and the biophase, respectively, and k_a and k_b the rate constants for its penetration into and escape from the biophase. R is free receptor and RD receptor-drug complex. k'_1 to k'_n are rate constants for enzymatic reactions which convert D_b to an inactive product in the biophase, or for any other processes, e.g., diffusion past intracellular barriers or storage, which make it unavailable for reaction with the appropriate tissue receptors. After washout of drug from an isolated tissue chamber, D_a rapidly drops to a negligible level and the duration of drug action is determined by k_b, k'_1 to k'_n, k_2, or some combination of these.

The above experiments demonstrated that inhibition of MAO can increase the time for half relaxation of contractions produced by phenylephrine (1×10^{-5} or 1×10^{-4}) to about 3 times that of controls. This indicates that, at the concentrations studied, the rate constant for deamination of phenylephrine by MAO is probably greater than the total of all the other rate constants for inactivation. Similarly, the combined rate constants for MAO and COMT appear to dominate the disposition of noradrenaline in concentrations of 1×10^{-6} and 1×10^{-7}. Inhibition of MAO increased the time for half relaxation after washout of lower concentrations of phenylephrine (1×10^{-6} and 1×10^{-7}), but to a lesser extent than inhibition of COMT plus MAO slowed the relaxation following contractions produced by similar concentrations of noradrenaline (Table IV). These results suggest that different rates of inactivation may provide at least a partial explanation for differences in the recovery times of smooth muscle structures contracted by different agonists and differ-

ences between structures for the same agonist. Agents such as methoxamine and amphetamine might owe their long duration of action after washout in vitro, not to parameters of the receptor-drug complex or to markedly lower k_b's, but to small or negligible rate constants k'_1 to k'_n, since they are not substrates for either COMT or MAO. The contributions of endogenous mechanisms of inactivation, which undoubtedly vary from drug to drug, certainly must be considered in the formulation of hypotheses in receptor theory.

Demonstration that the slow relaxation of aortic strips is related to the persistence of agonist in the environment of the tissue receptors makes it difficult to assess experiments in which the smooth muscle is forced to relax by the imposition of additional tension. Experiments where the outcome is sensitive to low levels of agonist from previous tests must be interpreted with particular caution; e.g., (1) Studies in which the presence or absence of blockade or potentiation are determined from sequential responses to a standard concentration of agonist. (2) Studies of threshold responses to a standard agonist interspersed between tests with other agonists. (3) Studies on the restoration of depressed responses to drugs such as tyramine in reserpinized tissues after incubations with noradrenaline.

These results also have implications regarding the location of α adrenergic receptors. The observation that noradrenaline persists in the environment of the receptors for long periods after the concentration in the bathing fluid has been reduced to zero indicates that the biophase in question is behind some important diffusion barrier. The most obvious barrier is the plasma membrane of smooth muscle cells, and

this possibility is strengthened by the observed effects of inhibiting the intracellular enzymes MAO and COMT, which indicate that the sympath-omimetics studied can readily penetrate cells.

A possible alternative explanation for the slow relaxation of aortic strips after exposure to a high concentration of agonist is that the very high gradient of amine results in its intracellular accumulation, and that after washout this store is gradually dissipated by movement outward past the receptors. There appears to be no positive evidence for such a mechanism and it is made less likely by the finding, reported below (Section V), that intracellular enzymes can play an important role in terminating the actions of even very low concentrations of sympatho-mimetic amines.

The observations reported above on the relaxation of aortic strips after washout of agonists indicate that only limited information on endogenous inactivation mechanisms can be obtained by this procedure. Inhibition of enzymes capable of inactivating the agonists had a clear effect on the rate at which their action was terminated (rate of relaxa-tion) only when the agonist was present in relatively high concentration. However, these data do not prove that enzymatic degradation is less im-portant at lower, more physiological, concentrations, because the exis-tence and importance of endogenous mechanisms could only be determined relative to the effect of a competing outward diffusion gradient. The sudden drop in drug concentration in the external aqueous phase to a negligible level would establish a steep concentration gradient. This undoubtedly contributes to the termination of drug action, but its magni-tude and its variation with different agonists and concentrations could

not be determined with certainty.

It follows from the above considerations that the presence in tissues of pathways capable of inactivating a drug does not allow firm conclusions regarding their involvement or importance in terminating its action. Diffusion of agonist from receptor sites into intracellular compartments or into the fluid environment of the cell could play a variable role both in vivo and in vitro in the termination of drug action, and could also alter the manner in which a drug is presented to the other inactivating mechanisms. Mechanisms of disposition of possible physiological importance could also be obscured by the diffusion of drug into the bathing medium in in vitro studies. The investigation of tissue mechanisms for the inactivation of catecholamines and other smooth muscle stimulants required the development of a method for separating external diffusion from events within the tissue. The following chapter describes a technique which minimizes the role of diffusion by immersing drug contracted aortic strips in oil.

B. THE OIL IMMERSION TECHNIQUE

In experiments to be described in this section, strips of rabbit thoracic aorta were immersed in oil to prevent the escape of water soluble, relatively fat insoluble, drugs. The procedure was to produce a contraction with the desired stimulant while the strip was in an aqueous medium (Krebs) and, after equilibration, to drain the muscle chamber and replace the bathing medium with oil. The rate of inactivation of the "trapped" drug was then followed by recording the rate of isotonic relaxation of the strip.

The earliest use of oil in conjunction with living tissues is lost in antiquity. An appreciation of the inertness of various oils towards living tissues was apparent in prescriptions from Egyptian papyri (Leake, 1952), which describe the use of oil, presumably as a vehicle, for both local application and ingestion. The medicinal use of oil was held in such esteem by the ancient Egyptians that the Hearst papyrus offers a supplication to be recited, to insure its efficacy, on each occasion of its prescription. The inocuousness of oil was also recognized in the Middle Ages, when it was a common ingredient of prescriptions (Clendening, 1942), probably as a vehicle for the other ingredients.

The inertness of mineral oil is now routinely accepted in biological research. Nerves are commonly bathed in oil during the recording of action potentials and in this environment conduct impulses for many hours (e.g., Hodgkin, 1939; Gillespie, 1962). Similarly, the exposed cerebral cortex of the cat can maintain undiminished responsiveness for periods exceeding two days while covered with mineral oil (C. Pinsky, personal communication). Whole organs have also been immersed

in mineral oil for plethysmographic measurements (Thoenen et al., 1964).

The results presented below are directed to demonstrating the tolerance of rabbit thoracic aorta strips to even long periods of oil immersion, the absence of external loss of drug following oil immersion, and the suitability of this technique for the study of mechanisms of drug inactivation.

1. RESULTS

a. Behavior of Drug Contracted Aortic Strips in Oil:

A description of details of the oil immersion procedure was presented in the Methods section. Experiments were first performed to establish the feasibility of the technique. Aortic strips were contracted by angiotensin and by potassium chloride and the stimulants either washed out or the chambers drained and refilled with oil. Contractions and relaxations were recorded isotonically on a slowly-moving kymograph drum (1.4 mm/min.). Strips contracted by angiotensin relaxed in oil at a rate comparable to that in Krebs solution (Fig. 7a). Strips contracted by KCl relaxed in Krebs, but maintained their plateau height throughout the period of oil immersion, relaxing only after they were returned to the aqueous medium (Fig. 7b). These results suggested the presence of endogenous mechanisms capable of efficiently terminating the action of angiotensin. It has been previously reported that red and white blood cells and various tissue extracts such as those of liver and kidney contain angiotensinase activity (Erdös, 1962). Conversely, the failure of strips contracted by KCl to relax in oil reflected the absence of any

a.

b.
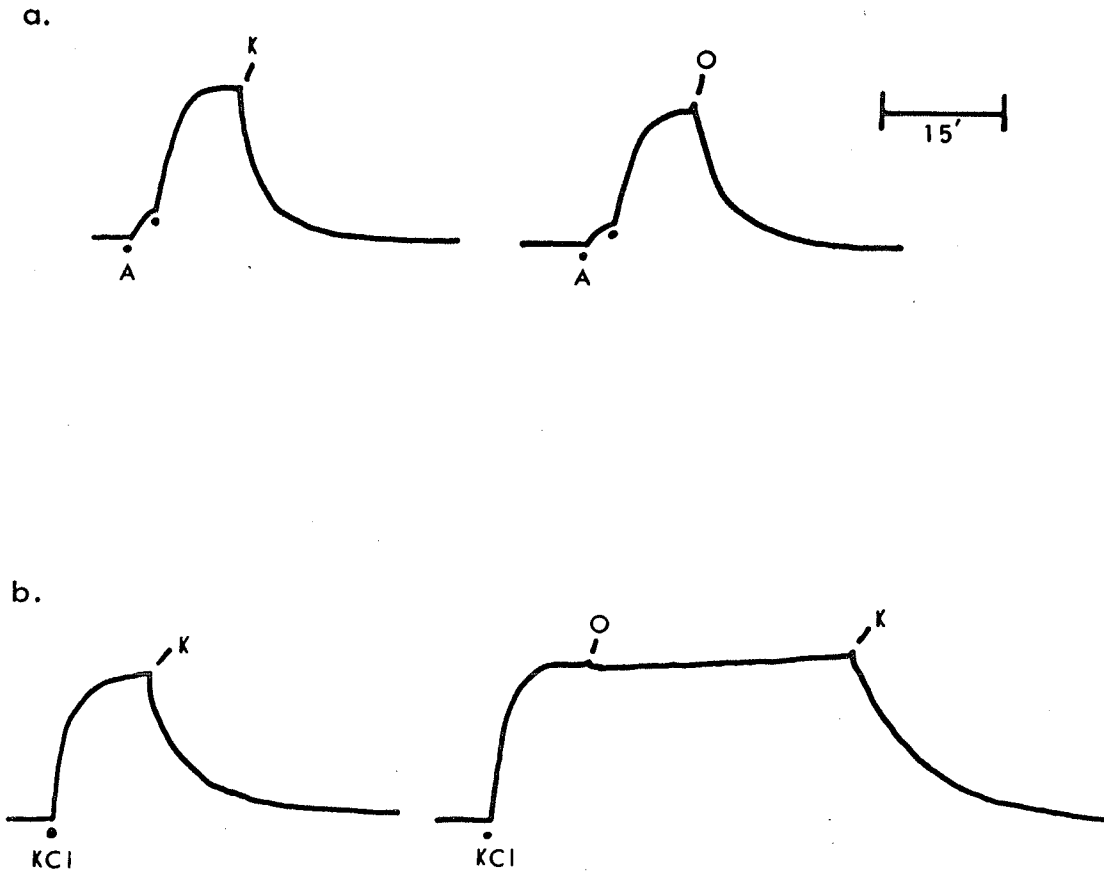
Fig. 7. Behavior of Aortic Strips Contracted by Angiotensin and by KCl in Krebs Solution and after Oil Immersion.

Record a. is from a strip contracted with angiotensin (A) (1 and 3 X 10^{-8}) and washed in Krebs solution (K) (left) or immersed in oil (O) (right). Record b. is from a strip contracted with KCl (0.1 M) and washed in Krebs solution (left) or immersed in oil for about 30 minutes and then washed in Krebs solution (right).

mechanism by which the tissue could inactivate the quantity of potassium presented to it.

b. Comparison of Various Oils:

Relaxation from equivalent contractions produced by noradrenaline were compared in peanut, mineral and silicone oils. Early experiments with peanut oil gave results comparable to those with the other two. However, it was later noted that peanut oil could include some material which produced a secondary contraction after partial relaxation of the muscle. Reproducible results were obtained with either mineral or silicone oil, and the rates of relaxation in the two media did not appear to differ. The former was selected for general use because it is much less expensive.

c. Effect of Oil on Contractile Responses:

Twelve aortic strips were contracted by noradrenaline (1 X 10^{-8}), and after washout and return to basal tone, 6 were allowed to remain quiescent in Krebs solution and 6 were immersed in oil for 60 minutes. About 30 seconds after the oil was replaced by Krebs solution the same concentration of noradrenaline was again added to the chambers and the response of each strip compared to its own initial response (Fig. 8). Responses to the first and second noradrenaline tests in each group of muscles are compared in Table V. Although the second responses were greater, the increases in peak and plateau heights were almost identical in the 2 groups. The fade from peak to plateau height of strips exposed to the oil increased by 5.5% of the peak height, compared to an increase of 2.0% in the control (Krebs) group. This difference was not significant at the 5% level, but may reflect a real effect of the oil. However,
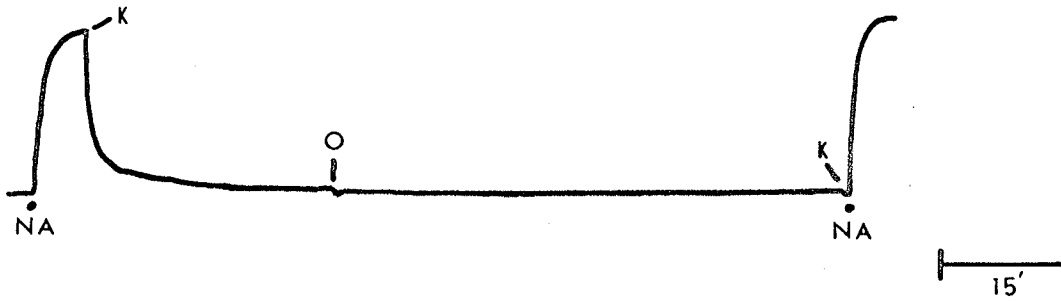
Fig. 8.   Effect of Prolonged Oil Immersion on Basal Tone and Contrac-
tility of an Aortic Strip.

Strip was contracted by noradrenaline (NA) (1×10^{-8}), and
after washout (K) and return to basal tone, was immersed in mineral
oil (O) for 60 minutes. Thirty seconds after return to Krebs solution
(K) it was recontracted by the same concentration of noradrenaline.
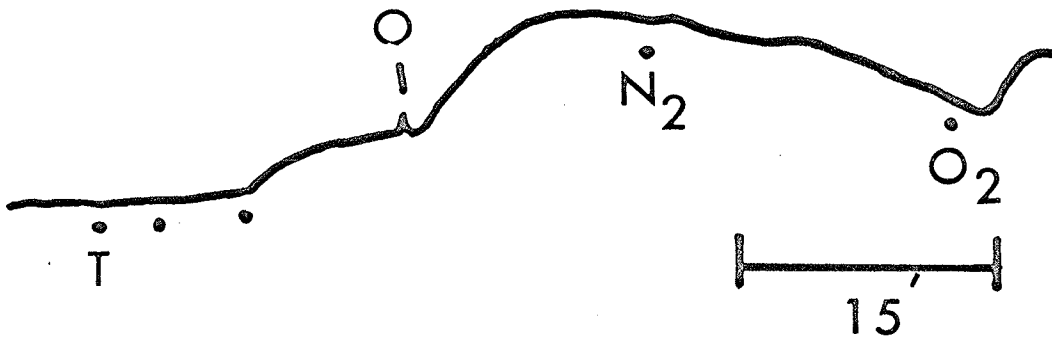
Fig. 9.   Effect of Oxygen Deprivation on a Tyramine Contracted Aortic
Strip in Oil.

Strip was pretreated with iproniazid and contracted by cumu-
lative concentrations of tyramine (T) (1×10^{-7}, 1×10^{-6}, 1×10^{-5})
(dots). Contraction normally increases somewhat after oil immersion
(O) and is then sustained for long periods. N_2 indicates point at
which N_2-CO_2 was substituted for O_2-CO_2 and O_2 the return to the stan-
dard aeration mixture.

TABLE V

EFFECT OF PROLONGED OIL IMMERSION ON CONTRACTILITY OF AORTIC STRIPS

Test	Peak Response (mm)		Plateau Response (mm)		Fade (mm)	
	Oil	Krebs	Oil	Krebs	Oil	Krebs
1st	30.7	25.7	30.2	25.3	0.5	0.3
2nd	35.5	29.2	33.0	28.2	2.5	1.0
% change	+ 15.8 ± 2.5%	+ 14.3 ± 5.3%	+ 9.3 ± 2.1%	+ 12.1 ± 4.8%	+ 5.5 ± 1.5%	+ 2.0 ± 0.75%
	N.S.		N.S.		P < 0.10 > 0.05	

Results from 6 pairs of strips, contracted twice by noradrenaline
(1×10^{-8}) After recovery from the first response, one of each
pair was immersed in oil for 60 minutes, while the other remained
in Krebs solution. Both were tested 30 seconds after replacement
of the oil by Krebs solution. See text for further details.

the fade is such a small part of the total contraction height that this would be of minor significance. Only a few strips showed a slight increase in basal tone, never exceeding a few millimeters, toward the end of the 60-minute period in oil. Other strips were kept in oil for as long as 120 minutes without any change in tone.

d. Oxygenation of Aortic Strips in Oil:

Adequacy of oxygenation in oil was tested on iproniazid pretreated strips exposed to tyramine (Fig. 9). Such strips usually maintain an undiminished contraction for at least 60 minutes. However, when the gas mixture was changed to 95% N_2 and 5% CO_2 from the regular 95% O_2 and 5% CO_2, the contraction amplitude began to decline slowly. This slow relaxation continued until the O_2-CO_2 mixture was reintroduced, when the contraction returned to near its original amplitude. The subsequent downward slope is probably evidence of some damage sustained during the period of anoxia. These results demonstrated an effective exchange of gases through the oil.

e. Effect of Oil Immersion on Responses to Drugs with Various Mechanisms of Action:

Aortic strips were contracted with noradrenaline (1 X 10^{-8}) and tested with cocaine (1 X 10^{-5}) before and after a period of oil immersion. No alteration in the degree of potentiation of the noradrenaline responses by cocaine was seen. Similarly, the responses of strips cut from a single aorta to tyramine, with and without prior oil immersion, did not differ appreciably.

Aortic strips contracted submaximally in Krebs solution can then either contract further, relax, or remain at a plateau height. It had to be clearly shown that strips under oil retained this dimensional

flexibility. Figure 10 shows the behavior in oil of 3 strips contracted with different sympathomimetic amines. The noradrenaline contracted muscle relaxed, the methoxamine contracted muscle retained approximately the plateau established before its immersion in oil, and the tyramine contracted muscle underwent further contraction before reaching a plateau. Explanations for these differences in behavior will be discussed in later sections of this thesis. These results demonstrated that muscles under oil have the same dimensional flexibility as in Krebs solution; they can contract, relax or maintain a plateau height.

f. Test of the Technique:

As a prelude to a detailed study of endogenous routes of inactivation of sympathomimetic amines, the nature of the data which could be obtained with the oil immersion technique was explored in a preliminary study of the effects of iproniazid pretreatment on the rates of relaxation in oil of strips contracted by dopamine and Cobefrine. The former has a terminal primary amine group and no β hydroxyl, and is an excellent substrate for monoamine oxidase (MAO). The catechol nucleus also makes it a substrate for catechol-O-methyl transferase (COMT). Cobefrine has an α methyl group, and is not a substrate for MAO but, like dopamine, it is a substrate for the transferase. The experiments with dopamine were performed on strips from reserpinized rabbits to eliminate possible complications from the noradrenaline releasing action attributed to this compound. Cobefrine, in the concentration used in these experiments, had no demonstrable indirect action.

Cobefrine (1×10^{-7}) and dopamine (1×10^{-6}) produced mean heights of contraction of 20.0 and 16.3 mm, respectively, before and
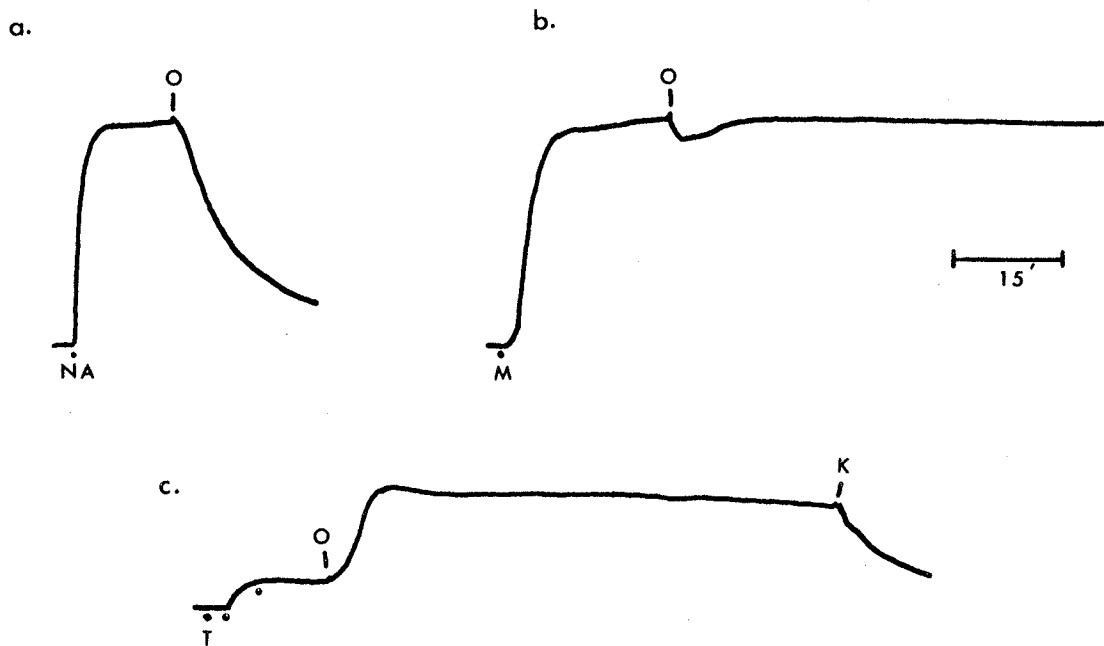
Fig. 10. Dimensional Flexibility of Responses of Aortic Strips after Oil Immersion.

a. Response of a strip contracted by noradrenaline (NA) (1×10^{-8}). b. Response of a strip from a reserpine pretreated rabbit contracted with methoxamine (M) (1×10^{-7}). c. Response of an iproniazid pretreated strip contracted by cumulative additions of tyramine (T) $(1 \times 10^{-7}, 1 \times 10^{-6}, 1 \times 10^{-5})$. Time of oil immersion is indicated by (O) and return to Krebs solution by (K). See text for detailed description of experiments.
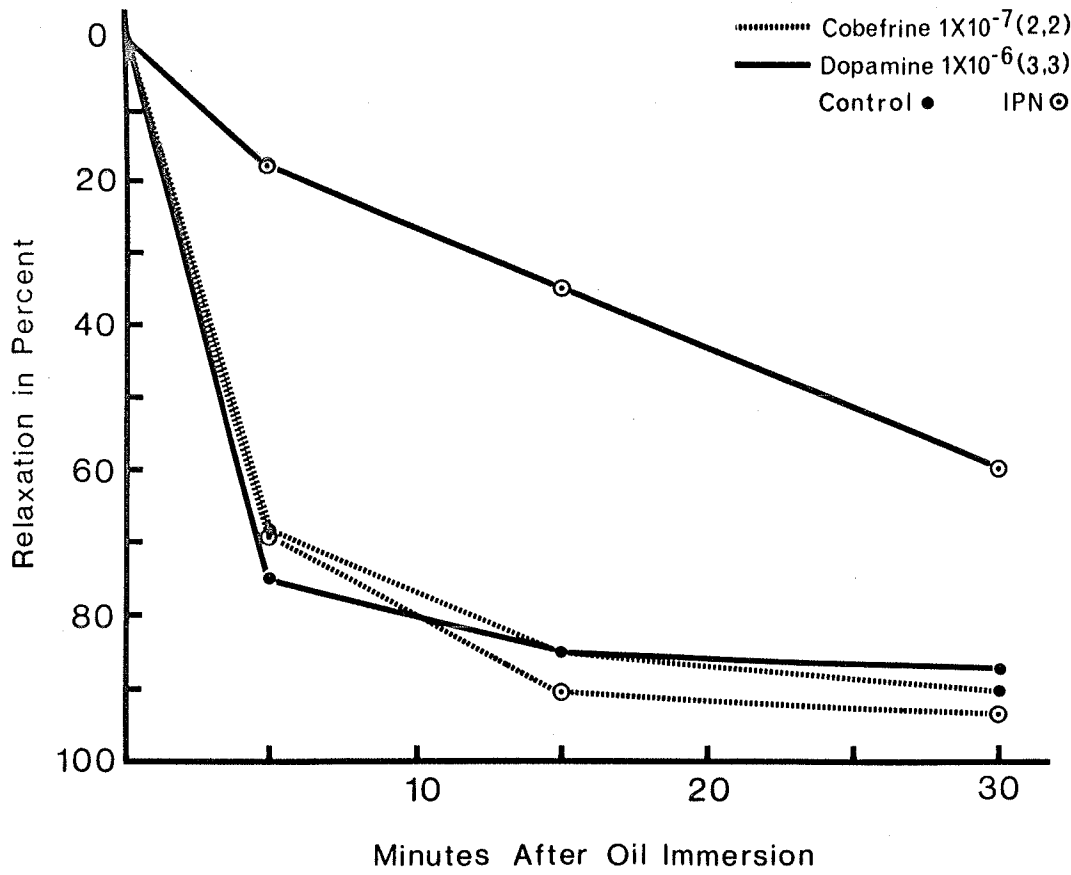
Fig. 11. Effects of Iproniazid (IPN) on Relaxation of Cobefrine and Dopamine Contracted Aortic Strips after Oil Immersion.

Figures in parentheses indicate the number of preparations represented by each curve.

15.5 and 26.0 mm after treatment with iproniazid. The MAO inhibition increased the time required for 50% relaxation of dopamine contracted strips from 3.5 to 24 minutes, but did not influence the rate of relaxation of Cobefrine contracted strips (Fig. 11). These results demonstrated that the major inactivation pathway for dopamine in aortic strips is deamination.

2. DISCUSSION

Major factors which complicate the interpretation of studies on specific tissue mechanisms for the inactivation of sympathomimetics and other agonists include:

(a) The need, in many cases, to expose the organ under study to an amount of drug far in excess of that required to produce a response in order to obtain sufficient material for biochemical or bioassay procedures. The results of such experiments may give an entirely erroneous view of the relative importance of various inactivation mechanisms at more realistic drug concentrations.

(b) The inability to present the organ under study with a fixed amount of drug with the assurance that changes in active drug level, metabolite production, etc. are due solely to endogenous mechanisms, and do not include the effects of exchange with the environment of the tissue.

(c) The laborious and tedious procedures often required to demonstrate individual pathways of metabolic inactivation and altered levels of active drug.

(d) The difficulty in relating alterations in tissue inactivation to altered responses of the organ under study, since these two often must be studied under radically different conditions.

The results described in the present chapter provide the basis for a method which permits the study of endogenous routes for the disposition of smooth muscle stimulants without the complications introduced by diffusion between the cells and their fluid environment, and with only that concentration of drug required to produce an easily measured response of the muscle under study. In addition, it allows alterations in contractile response (e.g., potentiation) and in inactivation to be studied in the same preparation. The method depends on the use of inhibitors of specific inactivating mechanisms to obtain information on their individual contributions to the overall process of drug inactivation.

Data are obtained by simple kymographic recording of the isotonic relaxation toward baseline of drug-contracted smooth muscle, spirally cut strips of rabbit aorta in the experiments described. Relaxation has been related to endogenous disposition of drug on the basis of:

(a) Demonstration that relaxation after washout of a smooth muscle stimulant is a function of the level of active drug in the environment of the specific tissue receptors for that drug (Section III, A).

(b) Elimination of diffusion between the tissue and its environment by replacing the aqueous bathing medium with oil after the tissue has reached equilibrium, or a steady state, with the drug or drugs to be studied.

The results reported above indicate that the relaxation of a

smooth muscle structure in oil can be used as a measure of endogenous

drug inactivation. This is supported by the observations that:

(a) The oil itself exerted no apparent pharmacological action.

(b) The oil did not appear to interfere with tissue function

as reflected in contractile performance. Reduction of the extracellular

space from an essentially infinite volume (10 ml) to a volume only

slightly greater than the interstitial space of the test tissue did not

appear to alter transmembrane balance of ions and nutrients, gas ex-

change, pH or other factors to an extent demonstrable in the performance

of the tissue.

(c) The selective prolongation of the relaxation time of dop-

amine but not that of Cobefrine contracted aortic strips in oil by pre-

treatment with iproniazid indicated that the technique is capable of

assaying the activity of specific endogenous inactivation mechanisms.

However, generalizations from the results of studies with the

oil immersion technique require a full appreciation of the differences

between this and other test systems. The termination of drug action

in oil cannot be equated directly with termination of drug action in

vivo, or in an isolated tissue in an aqueous medium. The oil immersion

system intentionally minimizes diffusion into the environment of the

tissue so as to focus on endogenous mechanisms of drug inactivation,

their identification, and the evaluation of their relative contributions.

Thus, in oil, termination of action is equivalent to endogenous inactiva-

tion. In contrast, preparations of isolated smooth muscle in an aqueous

medium probably maximize diffusion into the extracellular fluid as a

mechanism for the termination of drug action. The bath concentration

quickly drops to negligible levels at washout and repeated changes of bath fluid assure maintenance of a maximal concentration gradient out of the tissue. Thus, in an aqueous medium in vitro, termination of action is determined by diffusion plus a variable contribution of endogenous mechanisms.

The termination of drug action in vivo is much more difficult to analyze. The role of diffusion out of a tissue is affected by variations in extracellular concentration of drug, which are in turn modified by blood levels, regional blood flows, sinks of metabolism, nerve activity (in the case of chemical mediators), etc. Although these factors are difficult to evaluate quantitatively, their net effect probably is to make the contribution of outward diffusion to the termination of drug action in vivo less than in the case of aqueous systems in vitro. Inasmuch as alternative mechanisms for the termination of drug action are competitive, many factors such as the manner of presentation of a drug to a tissue, its local and average concentration, etc. could cause wide, and perhaps transient, variations in the contributions of various inactivation mechanisms, which at present are not determinable under physiological conditions in vivo.

IV. HISTORICAL INTRODUCTION - THEORIES TO EXPLAIN
TERMINATION OF THE ACTION OF THE
SYMPATHETIC MEDIATOR

Innumerable theories have been proposed over the years to explain the termination of the action of the sympathetic nerve mediator, whether circulating in the blood or released locally. Most investigations have relied on information obtained from studies with injected amine to provide clues to the disposition of mediator released by nerve activity. The hazards of such a translation have become increasingly more obvious as the experimental methods and approaches have become more sophisticated.

A most difficult obstacle to a proper understanding of the mechanisms responsible for terminating the action of the sympathetic mediator has been the reluctance of proponents of various theories to make a clear distinction between ultimate fate and termination of action. These events have very different physiological significance, and there is a gradually increasing awareness among investigators that the many mechanisms which may participate in the final inactivation of the mediator makes the terminating mechanism difficult to identify. It is interesting to note that although a wide variety of sophisticated research techniques has been applied to this problem, the strongest evidence for the current dominant theory has been obtained with a pharmacological tool -- cocaine.

This historical introduction reviews published work which is pertinent to theories regarding the mechanisms which terminate the action of catecholamines and attempts to relate them to current research in this field.

1. DIFFUSION AWAY FROM THE SITE OF ACTION THROUGH EXTRACELLULAR FLUID

The plausibility of disappearance from its site of action of at least a portion of the catecholamine released by nerve activity by escape through the extracellular fluid into the blood stream has been acknowledged in most termination of action theories. In fact, the first demonstration of chemical mediation of sympathetic nerve activity (Loewi, 1921) was only possible because stimulation of the accelerator nerves to the frog heart resulted in an escape of mediator which excited a second heart perfused by the same fluid. Cannon and Rosenblueth (1937) studied effects of mediator which reached the general circulation after stimulation of sympathetic nerves to individual effectors as a basis for their theory of the two sympathins. Paradoxical pupillary dilatation in the sympathetically denervated eye of an adrenalectomized cat, concomitant with excitement, was explicable only on the basis of sympathetic mediator escaping into the general circulation and contracting the hypersensitive radial muscles of the denervated iris (Cannon and Rosenblueth, 1949).

Despite these early convincing demonstrations that transmitter can diffuse away from its site of release, present theories dismiss diffusion as a minor, if not negligible, factor in the termination of transmitter action. The discovery of specific mechanisms capable of inactivating adrenaline and noradrenaline (e.g., catechol-O-methyl transferase) shifted the focus of interest away from simple diffusion, even before this was warranted by experimental evidence. However, important evidence against this mechanism soon followed. A group of Swedish workers convincingly demonstrated that the normal upper limit of impulse discharge

in the thoracolumbar (sympathetic) nerves is 6 to 8/sec. (See Celander, 1954.) They implied that earlier studies showing overflow of mediator into the blood stream were performed with unphysiological rates of stimulation. This was confirmed by Brown and Gillespie (1957). These workers found, using the blood perfused cat spleen in situ, that stimulation of the splenic nerves at frequencies below 10/sec. yielded no assayable quantity of noradrenaline in the venous outflow. Similar negative results were obtained by Thoenen et al. (1964) when the nerves to a spleen perfused with McEwin solution in vitro were stimulated at rates below 6/sec.

However, von Euler et al. (1954), and many others, have reported a daily output of noradrenaline in normal human urine which is unaffected by adrenalectomy. It can only be assumed that this represents a trickle of mediator which has reached the blood stream.

The termination of action of injected noradrenaline and adrenaline has also been considered as possibly occurring by diffusion into the blood. This is envisioned as a blood to tissues to blood movement in which declining blood levels of amine would allow a concentration gradient outward from tissues to terminate the action. (See Elliot, 1905, for discussion; also Starling, 1915.) Such a theory obviously requires a far greater total rate constant for the inactivation of amine in the circulation than for that in tissues. This theory was prematurely relinquished when Welch (1934), and others, showed that constituents of blood actually stabilize adrenaline. It might have had a longer life if it had been appreciated that a major organ like the liver, with a high rate of amine inactivation, could act as a sink for circulating catecholamines.

2. PENETRATION OF EFFECTOR CELLS

Elliot (1905) may be considered the father of this hypothesis because of his statement that "adrenalin disappears in the tissues which it excites." This broad declaration on the fate of injected adrenaline permits him priority on any theory postulating termination of action in the vicinity of the effector cells. But the impression gained by this writer is that Elliot considered diffusion into parenchymal cells, followed by destruction, as the terminating mechanism.

A formal attempt to develop a termination of action theory based primarily on a movement away from the region of the receptors, into effector cells rather than into the extracellular fluid, was made by Blaschko (1954, 1956). He believed that monoamine oxidase (MAO) was involved in the inactivation of noradrenaline, and since it is an intracellular enzyme, catecholamines would need to penetrate cell membranes to reach the locus of inactivation. This passage from the site of action into cells was suggested as the mechanism terminating the action of noradrenaline. He also pointed out that an agent which interferes with entrance into cells could mimic enzyme inhibition and potentiate the action of noradrenaline by maintaining a higher concentration in the vicinity of receptors. Koelle (1958) also presented an interesting model which suggested that the action of the sympathetic mediator might be terminated by penetration into effector cells. He suggested, as had Blaschko, that some agents might potentiate responses to noradrenaline by interfering with this penetration.

At the time the above theories were proposed there was no direct evidence that catecholamines can penetrate cells, except the ob-

servation of Bain et al. (1937) that blood cells can inactivate circulating adrenaline by binding or absorption, which demonstrated that non-nervous cells can accumulate catecholamines.

At the present time no drug is accepted as potentiating responses to noradrenaline by interfering with its entry into effector cells because such entry is not considered to play any significant role in terminating the action of either endogenous or exogenous noradrenaline.

3. MONOAMINE OXIDASE (MAO)

Schmiedeberg (1877), after finding that benzylamine administered orally was excreted in the urine as the deaminated, glycine conjugated derivative, hippuric acid, presciently proposed that monoamino bases which contain the $-CH_2-NH_2$ grouping are broken down in the organism with the liberation of ammonia. Over thirty years later, Ewins and Laidlaw (1910), intrigued by observations indicating that tyramine had physiological effects similar to those of adrenaline (Dale and Dixon, 1909), as well as by its then recent demonstration in tissue extracts and ergot, studied the fate of this compound in dogs. They found that it produced marked sympathomimetic effects when administered by mouth and that a deaminated metabolite appeared in the urine. They also found that p-hydroxyphenylacetic acid appeared in oxygenated Ringer's solution containing tyramine which was recirculated through isolated cat or rabbit liver or rabbit uterus. Evidence for the deamination of tryptamine was presented by these same workers a few years later (Ewins and Laidlaw, 1913).

Investigators at the time considered the deamination process to be a nonenzymatic, hydrolytic replacement of an amino by a hydroxyl group, with subsequent oxidation of the alcohol to an acid. The first evidence that an enzyme was responsible for the deamination process was presented in 1928. Mary Hare demonstrated that cell-free extracts of rabbit liver deaminated tyramine, with an uptake of one atom of oxygen per molecule of tyramine, in the presence of cyanide. The enzyme was neither amino acid oxidase nor diamine oxidase, and was named "tyramine oxidase". Because of its high activity in the liver, Hare suggested that the enzyme served to protect the animal from intoxication by tyramine formed by bacterial fermentation in the intestinal lumen.

During these years, the disposition in the body of the supposed sympathetic mediator, adrenaline, was unknown. Study of its disposition was impractical because of the enormous doses required to allow assay of metabolites by the techniques then available. Ewins and Laidlaw gave their dogs half a gram of tyramine and perfused isolated organs over a period of hours with 150 to 500 mg quantities to provide sufficient material for assay. The administration of similar doses of adrenaline to a whole animal or perfused organ was clearly not feasible.

Adrenaline was known to lose activity very rapidly in vitro, due to autoxidation and subsequent polymerization, and some investigators assumed that the transience of responses to this agent in vivo was due to the same type of inactivation. Others, however, had observed that adrenaline is protected from autoxidation by tissue extracts and body fluids. Oliver and Schäfer (1895) showed that small amounts of suprarenal extract retained their activity for a longer time in blood than in

water or in solutions of inorganic salts. Welch (1934) reported that glutathione and ascorbic acid, which are normal constituents of blood, liver and other tissues, inhibited the oxidation of adrenaline. He considered it unlikely that inactivation in vivo could occur by autoxidation and suggested, instead, the involvement of specific enzyme mechanisms.

The first evidence that adrenaline could be inactivated enzymatically was provided by Blaschko et al. (1937a). By combining manometric determination of oxygen consumption with bioassay of residual adrenaline activity, they found that crude extracts of kidney and liver from various species contained a thermolabile, nondialyzable material which caused the uptake of one atom of oxygen per molecule of adrenaline inactivated. Blaschko and his coworkers cautiously suggested that the "adrenaline oxidase" system might play some role in the inactivation of adrenaline in vivo, since it was present in those organs considered responsible for its removal from the circulation. Richter (1937) soon reported that methylamine and an aldehyde were the products of this oxidative deamination reaction. In the same year it was recognized that this enzyme, and another monoamine deaminating enzyme (Pugh and Quastel, 1937a), were the same as the tyramine oxidase of Hare (Pugh and Quastel, 1937b; Kohn, 1937; Blaschko et al., 1937b, 1938). Eventually, the enzyme was called "monoamine oxidase" (MAO) to differentiate it from diamine oxidase.

Blaschko's suggestion that MAO might contribute to the inactivation of adrenaline seemed to provide an explanation for both the transient action of the sympathetic mediator and the potentiation of responses to sympathomimetic amines by diverse agents. Strategic placement of the enzyme, like cholinesterase in cholinergic systems, could permit it to

play an important role in terminating the action of the sympathetic med-
iator. If agents which inhibit cholinesterase could so dramatically po-
tentiate responses to acetylcholine, it seemed reasonable to suppose
that agents known to potentiate responses to adrenaline acted by inhibi-
ting MAO. For example, after Blaschko et al. (1938) reported that ephe-
drine inhibited MAO, Gaddum and Kwiatkowski (1938) found that this agent,
already known to potentiate the responses of a variety of preparations
to adrenaline, sensitized the cat's nictitating membrane, the frog's
heart and the vessels of the rabbit's ear to the effects of both adrena-
line and stimulation of sympathetic nerves. Ephedrine also increased
the yield of an "adrenaline-like" substance liberated into the venous
effluent from the rabbit ear after sympathetic nerve stimulation. Gad-
dum and Kwiatkowski attributed both the sensitizing action of ephedrine
and the increased venous output of transmitter to inhibition of MAO, and
suggested that the enzyme plays a key role in terminating the action of
both injected adrenaline and that released by sympathetic nerve activity.
Some workers, however, did not accept this view. Kohn (1937) and Rich-
ter and Tingey (1939) dismissed such a physiological role of MAO because
of the low affinity of the enzyme for adrenaline in vitro.

A few years later Philpot (1940) reported that 0.017 M cocaine
inhibited by 80% the monoamine oxidase activity of guinea-pig liver sus-
pensions. This finding was interpreted as further evidence that the
enzyme was located close to the sites of release and action of the sym-
pathetic transmitter, since cocaine was known to potentiate responses to
sympathomimetic amines. Philpot replied to the critics of this role of
monoamine oxidase that the in vivo affinity of the enzyme for adrenaline

could not be predicted from in vitro experiments because MAO is an in-

soluble enzyme whose activity depends on its orientation in the cell.

Many subsequent attempts were made to implicate MAO in adren-

ergic mechanisms. The incentive was either to strengthen the hypothesis

that MAO is the key factor terminating the actions of sympathomimetic

amines, or to provide an explanation for an otherwise inexplicable po-

tentiation. Examples include potentiation of responses to catecholamines

by methylene blue (Philpot and Cantoni, 1941), by antihistamines (Tick-

ner, 1951) and by subblocking concentrations of ergotoxine, piperoxan

and yohimbine (Jang, 1941).

Burn and Robinson (1952) made an impressive attempt to fortify

the hypothesis that MAO plays a major role in terminating the action of

catecholamines in reporting that MAO activity declined in the denervated

nictitating membrane, iris and foreleg vessels of the cat coincident

with the development of supersensitivity to adrenaline and noradrenaline.

Advocacy of MAO as the mechanism terminating the physiological

actions of adrenaline and noradrenaline reached a peak with the important

work of Schayer (1951). Using adrenaline labelled with C^{14} either in or

β to the methylamine group, he found that after intravenous administra-

tion of doses ranging from 0.09 to 0.30 ug/gm, cleavage occurred some-

where between the two positions. Schayer concluded that as much as 50%

of the injected adrenaline was deaminated by MAO.

It was reported in 1952 that iproniazid (Marsilid) could al-

most completely inhibit the MAO activity of several different tissues,

both in vitro (Zeller et al., 1952) and in vivo (Zeller and Barsky, 1952).

These authors suggested that iproniazid "may well become of the same im-

portance for the study of the adrenergic system as has eserine in the
analysis of the cholinergic systems." Schayer and Smiley (1953) found
that after treatment with this long-acting MAO inhibitor, almost all of
the injected labelled adrenaline was excreted in the urine with the N-
methyl group intact. Schayer and Smiley concluded that this confirmed
the major role of MAO in the metabolism of adrenaline in intact animals.

Bacq (1949) and Blaschko (1952), however, could not support
the position which attributed potentiation of responses to catecholamines
by cocaine and ephedrine to inhibition of MAO. They found marked dis-
crepancies between the doses of these drugs, and of a series of local
anesthetics, needed to potentiate responses to adrenaline in vivo and
those which inhibited the enzyme in vitro. Even more inconsistent was
the ability of ephedrine and cocaine to potentiate responses to Cobefrine
(Jang, 1940), although it is not a substrate for MAO.

Since a major aspect of pharmacology is the development of
concepts based on information obtained from drug studies, it is not
unusual for the growth and demise of theories to be associated with the
discovery and exploitation of a pertinent agent. The discovery by
Zeller et al. (1952) that iproniazid is a potent inhibitor of MAO with
a very persistent action began the demise of many aspects of theories
relating MAO to adrenergic mechanisms.

Greisemer et al. (1953), Balzer and Holtz (1956) and Corne
and Graham (1957) reported the failure of enzyme inhibiting doses of
iproniazid to potentiate pressor and nictitating membrane responses to
adrenaline and noradrenaline. Furchgott (1955) reported that a concen-
tration of iproniazid which completely inhibited the MAO activity of

aortic strips did not alter their responses to noradrenaline, adrenaline, acetylcholine or histamine, but did markedly potentiate those to tyramine. Celander and Mellander (1955) found that inhibition of MAO did not affect the arteriovenous difference in the concentration of adrenaline or noradrenaline infused into the spleen and skeletal muscle vascular beds, and Brown and Gillespie (1957) reported that the output of noradrenaline in the venous blood after stimulation of the cat splenic nerves was unaltered by iproniazid.

Evidence against MAO being a major factor in terminating the action of the sympathetic mediator obtained without the use of iproniazid also continued to accumulate. Armin et al. (1953) reported that denervation did not decrease the MAO activity of the rabbit ear, despite the markedly increased sensitivity of its vessels to adrenaline and other substances. Burn et al. (1954) withdrew the hypothesis proposed earlier by Burn and Robinson (1952) which ascribed denervation sensitization to a loss of MAO, because they were unable to confirm that sympathetic denervation decreased MAO activity in the iris and foreleg arteries of the cat. Koelle and Valk (1954), using a histochemical technique, found no selective association of MAO with adrenergic neuroeffector systems in the cat, although in the rabbit, adrenergic neurones had higher concentrations of MAO than did sensory or cholinergic neurones.

A comparative study of iproniazid and isoniazid, a congener which does not inhibit MAO, cast doubt on the significance of all reports claiming potentiation of responses to adrenaline and noradrenaline by inhibition of MAO (Kamijo et al., 1956). They found that high doses of both agents potentiated responses of the cat's nictitating membrane

to sympathetic nerve stimulation and to intra-arterial injections of adrenaline and noradrenaline, and that isoniazid potentiated and both iproniazid and isoniazid could reversibly inhibit the contractile responses of the isolated rat seminal vesicle and guinea pig uterus to adrenaline.

Disenchantment with the concept that MAO plays a major role in adrenergic mechanisms was evident in the writings of many workers by 1959 (Koelle, 1958, 1959; Zeller, 1959; Davison, 1958). However, although the enzyme appeared not to have a dominant role in terminating the action of endogenous and exogenous catecholamines, it could not be ignored. MAO clearly is involved in the ultimate metabolism of these amines (Schayer, 1951, Schayer and Smiley, 1953; Kopin and Gordon, 1962, 1963), and its inhibition increases the endogenous levels of noradrenaline in guinea pig, rat and dog hearts. (See Kopin, 1964.) In addition, the vasopressor response to sympathetic mediator released by reserpine is potentiated by MAO inhibition (Chessin et al., 1957), demonstrating that under certain circumstances, using potentiation as a guide, MAO may be involved in the termination of mediator action.

The still growing recognition that termination of action and metabolic fate are not synonymous terms, but may sometimes coincide, helped to provide a basis for the current belief that MAO does not play a major role in the inactivation of catecholamines, but does function to deaminate their O-methylated metabolites. In the individual neuroeffector system, MAO has been relegated to the function of regulating the intraneuronal level of amine. The evidence on which these views are based will be presented in succeeding sections of this Historical Introduction.

a. Distribution:

MAO is widely distributed in the animal kingdom (Blaschko,
1952; Blaschko and Hope, 1957). In the mammal it is found in glandular,
smooth muscle and nervous tissue. The liver, kidney, brain, intestines,
placenta and blood vessels are usually rich in enzyme, while it is ab-
sent from erythrocytes and plasma and negligible in skeletal muscle
Thompson and Tickner, 1951; Davison, 1958). Because nerves constitute
a negligible fraction of total cellular mass, a preponderance of the MAO
in most organs is located in nonnervous tissues.

Most workers consider MAO to be predominantly, if not entirely,
a mitochondrial enzyme (See reviews by Blaschko, 1952; Davison, 1958.)
The enzyme has been found in the particulate fractions of a large number
of tissues, but most detailed subcellular localizations have been done
on liver and brain. Baudhuin et al. (1964) found the enzyme in rat
liver to be almost entirely particle-bound. The mitochondrial fraction
contained about 70% and the microsomal fraction about 24% of the total
activity. The authors attributed the presence of the remainder in the
supernatant, soluble fraction to leakage from damaged particles.

b. Reaction Mechanism and Specificity:

MAO acts on compounds with an amino group attached to a ter-
minal aliphatic carbon atom. The primary reaction is:

$$RCH_2NH_2 + O_2 + H_2O \text{ ----- } RCHO + NH_3 + H_2O_2$$

Zeller (1963) describes the reaction as involving initial removal of one
of the two α-hydrogens, possibly with covalent bonding of substrate and
enzyme. The substrate-enzyme complex then reacts with water to form the
corresponding aldehyde, with the release of ammonia. The hydrogen accep-

tor, meanwhile, reacts with oxygen to form hydrogen peroxide. The active

site of the enzyme is considered by Zeller to be an aromatic ring plus a

two-membered aliphatic chain.

Primary and methyl substituted secondary amines are readily

oxidized by MAO. In a mixture of adrenaline and noradrenaline, rabbit

liver MAO oxidizes noradrenaline the more rapidly (Blaschko, 1952).

Tertiary amines, and secondary amines with a substituent larger than

methyl, are oxidized much more slowly. Phenylalkylamines, and others,

in which the amino group is not attached to a terminal carbon atom,

e.g., ephedrine, Cobefrine and amphetamine, are not deaminated. A hy-

droxyl group on the carbon β to the nitrogen, as in adrenaline and nor-

adrenaline, lowers affinity for the enzyme.

5-Hydroxytryptamine (serotonin), metanephrine and normetan-

ephrine are all good substrates for MAO. Histamine is not a substrate

for the enzyme, probably because of the interfering heterocyclic ring,

but methylhistamine is believed to be deaminated by MAO.

c. Inhibitors:

The first highly effective inhibitor of MAO discovered was

the hydrazine derivative iproniazid, and many related compounds have

since been demonstrated to have similar actions. Inhibitors of this

type are thought to occupy the same sites on the enzyme as do substrates,

and after initial attachment by secondary forces, to form a covalent

bond with the enzyme. The reaction sequence is believed to stop at this

point, and inhibition by iproniazid cannot be reversed. Zeller (1963)

postulated that, since iproniazid appears to block MAO irreversibly, the

restoration of enzyme activity over a period of several days may be the

result of synthesis of new enzyme rather than of reactivation.

The in vitro inhibitory potency of α-alkylamines (e.g., amphet-amine) is low. Under standard conditions their k_m values are 10^{-3} to 10^{-2} M. However, phenylcyclopropylamines, which are closely related to amphetamine, are remarkably potent inhibitors of MAO.

4. CATECHOL-O-METHYL TRANSFERASE

Armstrong et al. reported in 1957 that 3-methoxy-4-hydroxyman-delic acid was a metabolite of noradrenaline and, probably, of adrena-line found in human urine. The amount in the urine could be increased by the parenteral administration of noradrenaline or the oral administra-tion of 3,4-dihydroxy-DL-mandelic acid, and was very high in the urine of patients with pheochromocytoma. The authors suggested that methyla-tion of a phenolic group occurred in the body after deamination of nor-adrenaline by monoamine oxidase (MAO).

Later in the same year Axelrod (1957), acting on the possibil-ity that methylation might precede deamination, looked for and found an enzyme system in the soluble fraction of rat liver which catalyzed the O-methylation of adrenaline and of some other catechols. Either ATP and methionine or S-adenosylmethionine was required for activity, the liver enzyme apparently transferring the methyl group from methionine to the catechol nucleus. In the years 1958 to 1960, Axelrod and colleagues attempted to define the role of catechol-O-methyl transferase (COMT) in the metabolism of catecholamines. Normetanephrine (3-O-methyl noradrena-line) was identified in rat spleen and urine by paper chromatography,

and large amounts of metanephrine (3-O-methyl adrenaline) and normetan-
ephrine were found in the urines of patients with pheochromocytoma.

Axelrod (1959a,b) reported that after intraperitoneal adminis-
tration of tritium labelled adrenaline (0.2 mg/kg) to rats, 87% of the
radioactivity appeared in the urine within 24 hours, 54% as free and con-
jugated metanephrine. About 12% of the radioactivity was present as 3-
methoxy-4-hydroxymandelic acid and only traces as 3,4-dihydroxymandelic
acid. The metanephrine fraction was considerably increased in animals
pretreated with iproniazid. After the intravenous injection of H^3-adren-
aline (50 to 300 ug) in man, 90% of the radioactivity appeared in the
urine within 48 hours (Axelrod, 1960a). Metanephrine, both free and
conjugated, accounted for 34% and deaminated-O-methylated metabolites
for almost 51% of the administered activity. Less than 2% of the re-
covered material had undergone only deamination. By comparing the fate
of injected metanephrine with that of adrenaline, it was concluded that
about 70% of the administered catecholamine was initially O-methylated
to metanephrine (Axelrod et al., 1958; Axelrod, 1960a).

Early studies on the fate of injected noradrenaline and adren-
aline in the whole mouse indicated that almost all of the disappearance
of catecholamine during the first 5 minutes was due to O-methylation.
Pyrogallol, long known to potentiate responses to exogenous catechola-
mines and to sympathetic nerve stimulation, was found to reversibly in-
hibit COMT and to delay the disappearance of adrenaline and noradrenaline
in the whole mouse (Axelrod, 1960a). This author noted that 2 minutes
after the rapid intravenous injection of labelled adrenaline into cats
most tissues contained more H^3-metanephrine than H^3-adrenaline, and sug-

gested that COMT functions in many different tissues. On this basis,

Axelrod and his group proposed that the mechanism which terminates the

action of circulating adrenaline and noradrenaline, and probably that of

noradrenaline released at nerve endings, is O-methylation. MAO was re-

legated to the secondary role of deaminating these O-methylated metabo-

lites.

Although more recent work has validated Axelrod's claim that

COMT plays a major role in the metabolism of circulating noradrenaline,

and may even contribute to terminating its action, the emphasis has now

shifted from metabolic inactivation to other mechanisms as the major

processes terminating the action of mediator released at nerve endings.

a. Distribution:

COMT has been found in all mammalian species examined, includ-

ing man, monkey, cow, pig, rat, mouse, guinea pig, cat and rabbit, and

its tissue distribution is broad. Activity is consistently highest in

the liver, followed by kidney, intestine and most other glandular tis-

sues. It has also been found in blood vessels, spleen, myocardium,

white and red blood cells, brain, and in all peripheral nerves examined

(sympathetic, parasympathetic and somatic). It has been reported to be

absent from skeletal muscle and blood plasma. COMT is generally consid-

ered to be in the soluble fraction of cells (Axelrod, 1959a, 1965), in

contrast to MAO which is predominantly in mitochondria. A convincing

demonstration that COMT in peripheral organs is located predominantly

in nonnervous tissue is the finding of Potter et al. (1965) that com-

plete cardiac denervation by transplantation does not alter the level of

COMT in that organ.

b. Reaction Mechanism and Specificity:

COMT requires S-adenosylmethionine and Mg^{++}, although a number of other divalent cations can substitute for Mg^{++} in vitro. The metal is believed to function by linking the catechol hydroxyl groups to the enzyme. The active site is believed to include a sulfhydryl group. According to Axelrod (1959a), O-methylation proceeds by an interaction of the electrophilic methyl carbon of S-adenosylmethionine and a nucleophilic hydroxyl. Since the 3-hydroxyl groups of noradrenaline and adrenaline are strongly nucleophilic, the transfer takes place in this position. Catechols with a strongly nucleophilic hydroxyl in position 4 would presumably be O-methylated at this point.

All catechols are substrates for COMT, regardless of other substituents on the aromatic nucleus, monophenols are not. The enzyme shows no stereospecificity in respect to the d and l isomers of adrenaline.

c. Inhibitors:

Pyrogallol, the first COMT inhibitor studied, acts competitively, is a good substrate for the enzyme and is extremely labile. Belleau and Burba (1961) postulated from stereochemical considerations that the tropolone ring (2-hydroxy cycloheptatrienone) should be a good inhibitor of COMT. Their initial studies indicated that 4-methyltropolone was a more potent inhibitor than pyrogallol, was resistant to oxidation and acted noncompetitively. More recent studies by Mavrides et al. (1963) confirmed the potency of tropolones as COMT inhibitors both in vivo and in vitro, but these investigators concluded that they acted as competitive rather than as noncompetitive inhibitors of the enzyme. The nature

of the bond between the tropolone and the enzyme surface is unknown.

5. ADRENERGIC RECEPTORS AND UPTAKE BY ADRENERGIC NERVES

Brown and Gillespie (1956, 1957) found that the venous efflu-
ent from the blood-perfused cat's spleen in situ contained, after splenic
nerve stimulation, a vasopressor substance which was identified as nor-
adrenaline. It was barely detectable by their bioassay procedure when
the nerve was stimulated at a frequency of 10/sec., but was markedly
increased at 30/sec. The large increase in transmitter output per stim-
ulus at the higher frequency suggested that some endogenous mechanism for
disposing of noradrenaline had been saturated. These workers also found
that the α adrenergic blocking agents Dibenamine and phenoxybenzamine
markedly increased the output of noradrenaline at low frequencies of
stimulation, whereas that at 30/sec. was essentially unaltered. Indeed,
after α adrenergic blockade, the output per stimulus was equal at all
frequencies between 1 and 30/sec. Brown and Gillespie concluded that the
amount of transmitter liberated per stimulus within this range was con-
stant, but that at the higher frequencies local destruction was incom-
plete and noradrenaline spilled over into the bloodstream. They also
concluded that the mechanism for disposition of mediator released by
nerve stimulation involved the α receptors and was inoperative when they
were blocked.

The evidence on which Brown and his colleagues (1957, 1960)
implicated the α adrenergic receptors in terminating the action of the
sympathetic mediator was subjected to a different and equally unique

interpretation by W.D.M. Paton (1960). He noted that frequency of nerve stimulation and venous output of catecholamine were similarly related during the stimulation of splenic nerves and of splanchnic nerves to the adrenal medulla, and felt that the latter could not be related to the involvement of receptors since the adrenal gland releases amine directly into the bloodstream. He also noted that if output per stimulus were constant, the quantities of noradrenaline recovered by Brown and his co-workers indicated that endogenous disposition of amine was depressed at higher frequencies of nerve stimulation rather than that receptor saturation allowed an overflow of mediator.

Utilizing the then slender evidence that tissues can take up and store exogenous noradrenaline, Paton (1960), without experimental support, proposed that disposition of noradrenaline involves the amine releasing tissue itself, the nerve terminals. He said: "the sympathin is the dominant intracellular cation, like potassium in many other cells, released when the membrane potential is reduced, and sucked back, recovered, returned to store, when the events of excitation are over".

Paton's hypothesis that the action of the sympathetic mediator is terminated by its return to storage within nerves was seeded on fertile ground, and it is currently regarded by most workers in this field as accurately portraying the terminating event in adrenergic chemical mediation. Its enunciation was quickly followed by numerous and varied studies undertaken to demonstrate tissue uptake and binding of amines and to determine its locus and role in the inactivation of noradrenaline. Raab and Gigee (1955) had demonstrated that heart muscle could accumulate injected noradrenaline, but with doses far above the physiological range.

However, the synthesis of tritiated amines of high specific activity and the development of refined methods for the separation and assay of these compounds and their metabolites have recently made it possible to study the distribution and fate of noradrenaline and adrenaline after the administration of amounts much closer to those which probably are released by nerve activity.

In a direct comparison of the retention of noradrenaline and adrenaline by intact and denervated tissues, Hertting et al. (1961a) administered tritiated catecholamine intravenously to cats 5 to 21 days after unilateral superior cervical ganglionectomy. One hour after injection, the H^3-catecholamine content of structures innervated by the cervical sympathetic nerves (e.g., iris, salivary glands, etc.) were much lower in the chronically denervated than in the normally innervated tissues. They concluded that sympathetic nerves or associated dependent structures were responsible for the amine retention. Comparable differences between denervated and innervated salivary glands were found using unlabelled catecholamine (Strömblad and Nickerson, 1961). Similarly, Potter et al. (1965) found the amount of tritiated noradrenaline in transplanted (denervated) canine hearts to be only about 6% that of controls 15 minutes after injection.

Evidence was also obtained that exogenous, labelled catecholamine retained by tissues is sensitive to nerve activity. Hertting et al. (1962) gave cats tritiated noradrenaline intravenously and 30 minutes later denervated the superior cervical ganglion on one side. Twenty-four hours later the decentralized tissues (iris, salivary glands, etc.) appeared to contain more label than did contralateral controls. However,

only 5 animals were studied, and the results are not entirely convincing. In other experiments they found that long acting ganglionic blocking agents such as pempidine and chlorisondamine decidedly increased the amount of H^3-noradrenaline in rat hearts 4 hours after its administration.

Both reserpine (Hertting and Hess, 1962) and tyramine (Chidsey and Harrison, 1963) have been shown to increase the rate of release of both endogenous and preinjected H^3-noradrenaline from tissues, and similar increases have been reported after nerve stimulation. Rosell et al. (1963) demonstrated an increased concentration of tritiated noradrenaline in the perfusate from a skeletal muscle vascular bed during sympathetic nerve stimulation, and Hertting and Axelrod (1961) found a marked increase in effluent concentration of tritiated noradrenaline after splenic nerve stimulation. Chidsey and Harrison (1963) also showed that tritiated noradrenaline retained by the canine heart could be released into the coronary sinus blood by cardioaccelerator nerve stimulation as late as 48 hours after administration.

The fact that nerve stimulation and certain drugs (e.g., tyramine) can increase and nerve section or ganglionic blockade can decrease the release of retained exogenous noradrenaline is not absolute proof that the amine is stored in nervous tissue. If it were bound to "silent receptors" in the effector tissue, the release of endogenous amine might displace the labelled material, leading to an increase in its effluent and a decrease in its tissue concentration. Effector cell depolarization, and perhaps other concomitants of nerve activity might also release amine from nonnervous loci. Likewise, tyramine and similar agents might

displace amine directly from nonnervous loci, as they do from nerves.

However, convincing evidence that at least a portion of exogenous H^3-noradrenaline is retained in sympathetic nerves was obtained from morphological studies. Electron microscopic autoradiography of sections of rat pineal glands removed 30 minutes after a slow intravenous infusion of H^3-noradrenaline showed the radioactivity to be localized in areas of nonmyelinated axons containing granulated vesicles (Wolfe et al., 1962). The constant association of radioactivity with granulated vesicles suggested that the noradrenaline was retained in them. Wolfe and Potter (1963) reported similar findings in the spleen and cardiac atrium. A generally parallel distribution of endogenous and H^3-noradrenaline in subcellular fractions of rat heart, vas deferens and submaxillary glands was found by Potter and Axelrod (1963) up to 24 hours after intravenous administration of the label. The peak for both was in a "microsomal" fraction.

Marks et al. (1962) used a radioautographic technique to study the localization of tritiated noradrenaline in sections of the heart and spleen of the mouse. During the first few seconds after injection radioactivity was distributed throughout the cardiac parenchymal cells, but by one minute long thin fibers strongly labelled by the noradrenaline were found in sections of ventricle. The radioactivity was still present in the long fibers 4 hours after injection, when the more diffuse label had almost disappeared. These fibers appeared to lie outside of the myocardial cells, and their location was compatible with that of sympathetic postganglionic nerves. Similar observations were made on sections of spleen.

Using fluorescence microscopy, Norberg and Hamberger (1964) and Malmfors (1965) demonstrated that the entire adrenergic neurone (cell body, axon, and terminals) can accumulate exogenous noradrenaline. However, the physiological significance of these observations is difficult to assess. Uptake could be clearly demonstrated only after inhibition of MAO, and because quantitation was difficult in the presence of normal stores of catecholamine, most of the uptake studies were done on animals in which they had been depleted by reserpine.

Observations to date on the distribution of exogenous noradrenaline, of which those discussed above are representative, indicate that it can be taken up in many organs and retained for considerable periods. The highest concentration of amine taken up and retained unchanged (accumulated) appears to be closely associated with endogenous noradrenaline in adrenergic nerves. However, whether this component represents the major part of the total amine uptake by various organs, and its role in terminating the action of exogenous or endogenous noradrenaline are not clearly established.

6. CURRENT STATUS OF METABOLIC INACTIVATION AND NERVE UPTAKE

Any balanced assessment of adrenergic mechanisms requires a reconciliation of the concept that nerve uptake is the predominant fate of the sympathetic mediator with the optimistic reports implicating O-methylation as the major process terminating its action and the fact that despite its current relegation to a secondary role, MAO cannot be summarily dismissed. It is becoming increasingly apparent that a clear distinction

must be made between inactivation of the mediator (termination of action)
and its ultimate metabolism to an inactive derivative.

Early reports suggesting the dominance of O-methylation in the
inactivation of the sympathetic mediator were based on studies of the
fate of injected adrenaline and noradrenaline, mostly comparisons of
the amounts of different metabolites formed during various intervals
after administration. Evidence of the involvement of both binding and
metabolism in the disposition of injected catecholamines has come from
several sources. Whitby et al. (1961) killed mice at intervals after
the intravenous injection of 1 ug of H^3- noradrenaline and determined
the total content of labelled noradrenaline and its metabolites. Nor-
adrenaline fell to 0.5 ug and normetanephrine rose to 0.45 ug within 5
minutes. Thereafter the content of noradrenaline fell more slowly, and
0.16 ug was still present at the end of 6 hours. These results demon-
strated that the primary fate of the rapidly disposed of portion of nor-
adrenaline was O-methylation. However, a significant part was retained
in some form which protected it from enzymatic attack over a period of
at least 6 hours. Thus, binding seemed to be a considerably more im-
portant factor in the disposition of noradrenaline than had been sug-
gested on the basis of earlier studies with adrenaline (Axelrod, 1959a,
b). This difference between adrenaline and noradrenaline was confirmed
by Whitby and his coworkers, who found that 2 minutes after the intra-
venous injection of H^3-noradrenaline (25 ug/kg) the content of amine
was greater than that of metabolites in most tissues of the cat, where-
as the reverse was true after H^3-adrenaline. Crout et al. (1961) accep-
ted an important role of O-methylation in the metabolic fate of circula-

ting noradrenaline, but questioned the validity of equating this with the mechanism which terminates its action. Crout (1961) found that simultaneous inhibition of COMT and MAO only slightly potentiated cardiovascular responses of the dog to injected noradrenaline and suggested that tissue binding rather than metabolism may be the terminating mechanism.

Evidence that binding by tissues is important in the disposition of injected noradrenaline was provided by Strömblad and Nickerson (1961), who found accumulation in rat hearts and salivary glands one hour after the intramuscular injection of 1.0 mg of unlabelled amine to be of a magnitude (0.5 to 2.0 ug/gm of tissue) which suggested that specific uptake by tissues may be a major factor terminating the action of circulating amine.

Whitby et al. (1961) considered the finding of a high level of normetanephrine in a variety of cat tissues 2 minutes after the intravenous injection of H^3-noradrenaline (25 ug/kg) to be evidence that the metabolite was locally produced and, therefore, that COMT was also important in inactivating the noradrenaline released by nerves. Crout et al. (1961), however, took exception to any extension of the role of COMT not based on specific experimental evidence. In their opinion the tissue normetanephrine reported by Whitby et al. could have arisen by transport from the blood, where its level was high. They were also unconvinced that potentiation of responses to noradrenaline by pyrogallol defined the importance of COMT in local adrenergic mechanisms. Bacq had reported that this agent potentiated responses of normal and denervated cat nictitating membranes to injected adrenaline in doses of 20 to 30

and 2 to 10 mg/kg, respectively. (See Bacq, 1960.) Crout and associ-
ates found that the smaller doses produced little inhibition of COMT.

It soon became apparent that the local fate of noradrenaline
could only be studied in individual organs and could not be inferred
from the fate of circulating amine. Kopin et al. (1962) perfused iso-
lated rat hearts with H^3-noradrenaline (0.1 ug/min.) and assessed its
metabolism on the basis of metabolites found in the heart 12 minutes
after termination of the infusion, plus those released into the perfus-
ate. Normetanephrine, the deaminated metabolite, and the deaminated,
O-methylated metabolite accounted for 1.8, 1.0 and 1.2% of the infused
amine, respectively; 9.8% was retained unchanged. Thus, although O-
methylation was the major metabolic fate, tissue binding appeared to be
the dominant inactivating mechanism. Chidsey et al. (1963) labelled the
isolated dog heart with H^3-noradrenaline and found that 74% was extrac-
ted during a single passage through the myocardium. This was then
slowly released, mostly as metabolites; only 11.7 to 25% of the total
venous radioactivity was noradrenaline, whereas 39 to 62% was normetan-
ephrine. When they attempted to simulate sympathetic nerve activity by
administration of tyramine, noradrenaline increased to 41% of the total.
This indicated to these workers that enzymatic mechanisms were unable
to cope with the increased release, and they suggested that the same
situation might prevail during sympathetic nerve activity, when local
enzymatic mechanisms might become less important than other processes
terminating transmitter action.

The release of preinfused H^3-noradrenaline by nerve depolariza-
tion has also been studied. Rosell et al. (1963) found that 30 minutes

after infusion of H^3-noradrenaline into an isolated skeletal muscle vascular bed of the dog, 23% of the resting outflow of tracer was noradrenaline, and 22 and about 45% was normetanephrine and the deaminated, O-methylated product, respectively. Less than 2% was only deaminated. Stimulating the sympathetic chain at rates of 5 to 12/sec., while maintaining a constant blood flow, produced a marked, but delayed, increase in noradrenaline output and a concomitant marked drop in the total output of label. Following the termination of stimulation, total radioactivity increased and noradrenaline decreased to prestimulation levels. They concluded, as had Chidsey et al. (1963), that O-methylation was the predominant fate of spontaneously released amine, but not of amine released by nerve activity, which appeared to be inactivated either by diffusion into the blood or by tissue binding. Rosell and coworkers reasoned that under physiological conditions rebinding would be most likely because the released mediator would cause vasoconstriction and reduce blood flow. Hertting and Axelrod (1961) reported similarly that the cat spleen spontaneously release preinjected H^3-noradrenaline predominantly as normetanephrine, but that after sympathetic nerve stimulation at frequencies above the physiological range (10 and 30/sec.) the major labelled material in the effluent was noradrenaline. These authors also, without direct evidence, favored rebinding as the mechanism which, in competition with O-methylation and diffusion into the bloodstream terminates the action of noradrenaline released by nerve activity.

Another widely quoted attempt to evaluate the roles of MAO and COMT in the inactivation of noradrenaline released endogenously was that of Kopin and Gordon (1962, 1963). They infused tritiated noradrenaline

into rats over a period of 4 hours and determined the urinary excretion

of label over a 24 hour period. About two-thirds of the label was ex-

creted during the 3 hours immediately after the infusion, 23, 29 and

about 37% as noradrenaline, normetanephrine and the deaminated O-methyl-

ated metabolite, respectively. This initial output was considered to be

representative of the fate of noradrenaline which had not been bound to

tissues. The label present in the urine between 10 and 13 hours after

the infusion (4% of the total excretion in 24 hours) was considered to

be representative of the fate of bound amine which was slowly released

from nerves. Noradrenaline was only 6.4% and normetanephrine 9.5% of

this fraction. The deaminated metabolite was increased to 7.0% and the

deaminated, O-methylated to 61.5%. Kopin and Gordon also noted that

after reserpine administration the major urinary metabolites were deami-

nated, and inasmuch as the material released by this agent produced no

sympathomimetic effects, they postulated that a predominance of deamin-

ated metabolites was representative of mediator metabolized intraneuron-

ally and released in inactive form. It was been shown that after MAO

inhibition, sympathomimetic effects are prominent during catecholamine

release by reserpine (Chessin et al., 1957). Conversely, since the bulk

of the label released by DMPP, acetylcholine and tyramine appeared in the

urine as noradrenaline and normetanephrine, they considered the excretion

of these compounds to be the true index of sympathetic nerve activity.

Although Kopin and Gordon considered O-methylation to be the local meta-

bolic fate of noradrenaline released by nerves, they were careful to

acknowledge the possible dominance of tissue rebinding as the terminating

mechanism. Their experiments were only possible because of the prolonged

binding of infused H^3-noradrenaline, and presumably this binding could recur throughout the period of observation.

The conclusions drawn from most recent studies place tissue (sympathetic nerve) uptake of noradrenaline in a primary position as the mechanism terminating its action. However, direct proof of this is lacking, and it appears that this emphasis may be determined as much by the climate of opinion as by the data. In many cases very different interpretations are possible. For example, it could well be argued that "resting" output of noradrenaline and metabolites in venous effluents or urine is a better reflection of the steady-state disposition of mediator released under physiological conditions by tonic sympathetic nerve activity than is the material collected during transiently augmented release due to tyramine, DMPP, acetylcholine, or even nerve stimulation at or above maximal physiological frequencies. The latter could swamp local inactivating mechanisms or set up unequal tissue to blood gradients of materials with different mobilities, which might give a very distorted picture. Furthermore, it has not yet been clearly established that tritiated amine taken up by tissues and released by simulations of nerve activity is homogenous with and fully representative of endogenous catecholamine, or that tritiation does not alter interactions of noradrenaline with its metabolizing enzymes. It has been shown that α-deuteration of noradrenaline decreases its rate of oxidation by MAO (Belleau et al., 1961).

In summary, predominant current opinion holds that COMT is the major pathway for local enzymatic inactivation of noradrenaline. MAO is considered to be of minor importance in peripheral tissues, except intra-

neuronally, where it may contribute to regulation of the level of amine
stores and appears to inactivate noradrenaline released from storage
granules by reserpine. (See Kopin, 1964.) Circulating noradrenaline
is considered to be O-methylated and secondarily deaminated in the liver.

It has been established that many organs can take up and bind
noradrenaline, at least part of it in adrenergic nerves in a form which
can be released more or less in parallel with endogenous catecholamines
by nerve stimulation, tyramine, etc. Most workers in this field now
favor nerve uptake as the mechanism terminating the action of mediator
released by sympathetic nerve impulses. There is no direct proof of
this hypothesis, which is very dependent on the circumstantial evidence
that cocaine simultaneously potentiates responses to exogenous noradrena-
line and to sympathetic nerve stimulation, and inhibits the uptake of
noradrenaline by tissues. The tissue "uptake" mechanism blocked by co-
caine is believed to be at the nerve cell membrane, and to be responsible
for the return of extracellular amine to intraneuronal storage. The
actions of cocaine will be reviewed in detail in the next section.

7. EFFECTS OF COCAINE AND THE "UNITARY HYPOTHESIS"

Fröhlich and Loewi (1910) first reported that cocaine poten-
tiated certain responses to catecholamines, and subsequently it was
found also to potentiate responses to sympathetic nerve stimulation
(Rosenblueth and Rioch, 1933; Trendelenburg, 1959; Thoenen et al., 1964).
Tainter and Chang (1927) noted that cocaine qualitatively distinguished
between responses to tyramine and adrenaline, the latter being enhanced

and the former markedly reduced or abolished. This was labelled "the cocaine paradox" by Burn and Tainter (1931), who also reported that chronic sympathetic denervation caused very similar changes in responses to various sympathomimetic amines.

Most explanations of the action of cocaine have centered around the similarity of its effects to those of chronic sympathetic denervation (Burn, 1932; Fleckenstein and Burn, 1953; Fleckenstein and Stöckle, 1955). Both separate sympathomimetic amines into 3 general classes, those whose responses are: potentiated (Cobefrine, noradrenaline), basically unaffected (β-phenylethanolamine, ephedrine), and markedly diminished or obliterated (amphetamine, tyramine).

Demonstration that reserpine both depletes tissue stores of catecholamines (Carlsson et al., 1957) and markedly depresses responses to tyramine led to renewed interest in a possible action of cocaine on nerves. Burn and Rand (1958a, b) reported that depression of responses to tyramine by reserpine could be reversed by infusions of catecholamines, and Furchgott et al. (1963) found that cocaine, present during exposure of reserpinized tissues to noradrenaline but washed out before testing with tyramine, blocked this restorative action in vitro.

Burn and Rand (1958b) found that denervation and reserpinization similarly affected responses to various sympathomimetic amines, and both depleted tissue catecholamines. They suggested, as had Burn (1932), that agents whose effects are reduced by denervation or reserpinization act normally through the release of endogenous noradrenaline and adrenaline, and that the action of cocaine is to inhibit this release.

An explanation for the concomitant potentiation of responses

to catecholamines by cocaine, which is now widely accepted, is that only a portion of administered catecholamine acts on the adrenergic receptors; another portion is taken up and bound by tissues (MacMillan, 1959). If this uptake is blocked by cocaine, a larger amount will be available to act on the receptors, increasing (potentiating) the effect.

This basic hypothesis, that potentiation may result from diversion of agonist from storage to receptor activation, has been used to explain the effects of a number of drugs (Furchgott et al., 1963; Kopin, 1964, Malmfors, 1965; Isaac and Goth, 1965). Furchgott et al. (1963) proposed a unifying hypothesis to explain the cocaine paradox on the basis of observations on the interactions of noradrenaline, tyramine and cocaine on rabbit aorta strips and guinea pig atria in vitro. They postulated a specialized transport mechanism in the nerve cell membrane which moves extracellular noradrenaline into the nerve ending, where it is either stored or metabolized. Cocaine was assumed to block this transport, thus, diverting amine to the receptors, and tyramine was assumed to gain access to noradrenaline stores through the same transport process. They explained the observation that cocaine potentiated responses to noradrenaline just as well in reserpinized as in normal tissues by assuming that reserpine acts on intraneuronal storage of amine rather than on cell membrane transport. After reserpinization amine transported into nerves is assumed to be inactivated enzymatically rather than by storage. This facet of the proposed hypothesis is supported by the observation that intraneuronal accumulation of catecholamine in reserpinized tissues can be demonstrated by fluorescence microscopy only after inhibition of MAO (Malmfors, 1965). After sympathetic denervation, potentiation presumably is due to the absence of

the nerves into which transport normally occurs. This explanation of
the potentiating effects of cocaine and of denervation is entirely de-
pendent on the assumption that transport into nerves is normally a domi-
nant factor in terminating the action of the relevant amines.

Evidence that various organs can take up catecholamines was
discussed above. There also are numerous observations indicating that
cocaine can at least partially block the "uptake" of noradrenaline (e.g.,
Muscholl, 1961; Dengler et al., 1961), but relatively few have included
information on metabolites. The "uptake" measured is, therefore, the
amount of noradrenaline retained for a given period of time without
metabolic alteration.

Whitby et al. (1960) and Hertting et al. (1961b) gave cats
$dl-H^3$-noradrenaline (25 ug/kg), intravenously and measured tissue concen-
trations of the unchanged amine and of a metabolite, normetanephrine, 60
minutes later. Pretreatment with cocaine (5 mg/kg, intravenously) reduced
the concentration of tritiated noradrenaline in the liver, but left that
of normetanephrine unchanged, and reduced the concentrations of both in
the heart, spleen and adrenal glands. Plasma levels of H^3-noradrenaline
were elevated by cocaine for only 3 minutes after injection. These re-
sults are not entirely consistent with the hypothesis accepted by the
authors, that cocaine reduces uptake of noradrenaline entirely by inhi-
biting its accumulation in nerves. If cocaine acted only to block the
transport of amine into nerves, an increase in enzymatic destruction in
adjacent cells and, thus, elevated levels of normetanephrine would be
expected. The decreases observed cannot be considered secondary to de-
creased plasma levels of normetanephrine, because the concentration in

skeletal muscle was significantly elevated.

Further evidence that cocaine can block the uptake of noradrenaline by tissues, but in a manner not explicable on the basis of an effect limited to nerves was provided by van Zwieten et al. (1965). They found that cocaine (20 mg/kg, intravenously) given 15 minutes before intravenous administration of H^3-noradrenaline did not change either the plasma total radioactivity or H^3-noradrenaline levels, but did decrease normetanephrine significantly. It also decreased the total radioactivity and the H^3-noradrenaline in heart and spleen considerably. H^3-normetanephrine was decreased in the heart and unchanged in the spleen.

To reach the conclusion that cocaine causes a diversion of amine from storage in nerves to extraneuronal sites of metabolism, the authors calculated the tissue concentrations of metabolite as percent of total radioactivity. They did not report deaminated, O-methylated metabolite separately, but indicated that this was the difference between the values for noradrenaline plus normetanephrine and total radioactivity. This difference increased slightly, probably not significantly, in the spleen (17 to 22 muc/gm) and decreased in the heart (37 to 22 muc). These data do not indicate that cocaine increased the production of metabolites.

Observations on the effect of cocaine on local inactivation of noradrenaline released by sympathetic nerve activity is conflicting. Hukovic and Muscholl (1962) reported that cocaine approximately doubled the output of noradrenaline into the bathing medium after stimulation of sympathetic nerves to the isolated rabbit heart at frequencies of 10 to 20/sec. Similarly, Thoenen et al. (1964) found that cocaine in-

creased noradrenaline in the venous effluent from cat spleens from 40.6 to 70.6 ng in an 80 second collection period during and after stimulation of the splenic nerves at a frequency of 6/sec. for 10 seconds. Cocaine, infused at a rate of 10 ug/min. also increased the mean venous effluent concentration of noradrenaline from 2.2 ng/ml to 5.7 ng/ml when the spleen was perfused with a concentration of 8 ng/ml. A 10 times higher dose of cocaine caused only a slight further increase in effluent concentration, to 6.4 ng/ml. Kirpekar and Cervoni (1963), using a collection period of 40 seconds, found that cocaine (5 mg/kg) produced a small but significant increase in the outflow of noradrenaline associated with stimulation of the splenic nerves of cats. However, Blakeley et al. (1963) and Trendelenburg (1959) reported that cocaine (5-6 mg/kg) did not alter the venous output of noradrenaline, during collection periods of about 40 seconds, in response to stimulation of the splenic nerves at frequencies of 10 and 30/sec.

All of the investigators mentioned above assumed that any increase in effluent noradrenaline output produced by cocaine was due to inhibition of some nerve cell mechanism for disposition of the mediator, but none presented convincing evidence for this belief.

It is apparent that cocaine can both potentiate responses to catecholamines and decrease their uptake in certain organs. Whether these two parameters are causally related is less certain. MacMillan's (1959) suggestion that potentiation by cocaine is due to decreased tissue uptake, which allows a higher concentration of amine to reach the receptors was not based on any direct evidence. Trendelenburg (1959) reported that cocaine prolonged the pressor responses of spinal cats

to large (25 ug/kg) doses of noradrenaline given intravenously, and also increased the blood levels of noradrenaline attained, and concluded that delayed inactivation could fully account for the prolonged pressor response. A similar direct correlation between inhibition of uptake of injected noradrenaline by the heart and augmentation of the pressor response in rats was claimed by Muscholl (1961), who also reported that α-cocaine, atropine and the local anaesthetic, amethocaine, affected neither.

Evidence that increased plasma levels could not account for the potentiation of responses of individual effectors to injected noradrenaline was the failure of cocaine to potentiate responses of denervated organs in vivo and the wide variation in the degree of potentiation of responses of different effectors. In addition, Haefely et al. (1964) reported that cocaine potentiated responses of the nictitating membrane to close intra-arterial injections of noradrenaline, an effect which could not be due to increased circulating blood levels of the amine. However, some contribution of a systemic effect was observed by Eble (1964) who found that cocaine potentiated the vasoconstrictor action of noradrenaline in the blood-perfused hindlimb more when the sympathomimetic was administered intravenously than when given intra-arterially. He also found that the hindleg response to noradrenaline administered intravenously was greater when cocaine was given intravenously than when given intra-arterially.

Indirect evidence considered by many workers to support a causal relationship between the increased responses and the decreased tissue uptake of sympathomimetic amines produced by cocaine are the

findings that the ability of organs to bind noradrenaline is markedly depressed by chronic sympathetic denervation (Hertting et al., 1961a; Strömblad and Nickerson, 1961), and that cocaine does not potentiate responses of chronically denervated structures to noradrenaline (Kukovetz and Lembeck, 1961; Haefely et al., 1964; Furchgott et al., 1963).

Potentiation of responses to noradrenaline released by normal sympathetic nerve activity is probably partly responsible for the well-known "sympathomimetic" effect of cocaine, and potentiation of responses to sympathetic nerve stimulation by cocaine has now been shown in specific experiments on the nictitating membrane (Trendelenburg, 1959) and other organs. Haefely et al. (1964) found that cocaine potentiated responses of the nictitating membrane to nerve stimulation 20 to 30 fold and responses to injected noradrenaline 50 to 100 fold. Thoenen et al. (1964) reported that cocaine increased both the magnitude and the duration of splenic contractions induced by stimulation of its sympathetic nerves as well as the venous output of noradrenaline, and Hukovic and Muscholl (1962), and others, have reported that it increases responses to stimulation of the sympathetic nerves to isolated rabbit atria and increases the release of noradrenaline into the bathing medium.

Trendelenburg (1965) found that the relative magnitudes of potentiation of responses of the nictitating membrane to 3 catecholamines (l-noradrenaline $>$ l-adrenaline $>$ d-noradrenaline) by cocaine fell in the same order as did sensitization to these agents by denervation and their uptake by tissues. He concluded that this correlation supported the hypothesis that sensitization by cocaine is due to blockade of the uptake of the amines by nerves.

Although it seems to be generally accepted, the "unitary hypothesis" that cocaine potentiates responses to catecholamines because it inhibits inactivation by uptake into adrenergic nerves is based entirely on circumstantial evidence. This does not allow any firm conclusion as to whether the two observed effects, altered disposition and potentiation, are cause and effect, are both related in some less direct way to a single action of cocaine, or are expressions of two or more distinct actions of the drug.

8. ARROGATION BY THE "UNITARY HYPOTHESIS"

Despite the entirely circumstantial nature of support for the hypothesis which attributes potentiation by cocaine to an increased concentration of amine available to tissue receptors, secondary to inhibition of nerve uptake, the possibility of such a cause and effect relationship so captured the imagination of investigators dealing with adrenergic mechanisms that this principle has been extended to cover almost all known cases of potentiation of responses to sympathomimetics, also referred to as sensitization. As mentioned above, it is a major current explanation of denervation supersensitivity, and it is also used to relate the decreased nerve storage of catecholamines and the potentiation of responses to them by reserpinization (Brodie and Beaven, 1963; Kopin, 1964; Axelrod, 1965; Hertting, 1965). Although it was necessary to assume that the decreased storage after reserpine reflects only a difference in intraneuronal fate and not really decreased uptake in order to explain potentiation by cocaine after reserpinization (Furch-

gott et al., 1963), this obvious discrepancy has done little to dampen enthusiasm for this "unitary hypothesis".

Pertinent to the present discussion is the fact that inhibition of the "cocaine-sensitive mechanism" (nerve cell uptake) is now generally accepted as the explanation for the effects of β-haloalkylamines and other adrenergic blocking agents on amine disposition. Although Brown and coworkers attributed the increase in effluent output of noradrenaline following sympathetic nerve stimulation produced by Dibenamine and phenoxybenzamine to blockade of α adrenergic receptors (Brown, 1960; Blakeley et al., 1963). Paton (1960) interpreted their data on the basis of inhibition of reuptake by adrenergic nerves. This proved to be a highly popular suggestion and many workers have sought to demonstrate that the adrenergic blocking agents affect the same uptake mechanism blocked by cocaine, which is postulated to be involved in the transport of amines into sympathetic nerves. Dengler et al. (1961) incubated slices of cat tissues for 45 minutes in a Krebs-bicarbonate medium containing the desired drug and dl-H^3-noradrenaline (5 mug/ml). Uptake, calculated as the H^3-noradrenaline found in tissues in excess of that expected from diffusion equilibrium, in heart and spleen was inhibited 100% by cocaine, but Dibenamine had no inhibitory effect even in a concentration of 10^{-4} M. Uptake by spleen was inhibited 30% by ergotamine (10^{-6} M) and 50% by dichloroisoproterenol (5 X 10^{-5} M). In contrast, Hertting et al. (1961b) found that both cocaine and phenoxybenzamine inhibited uptake of noradrenaline in both heart and spleen. Other types of experiments have also produced conflicting results. Thoenen et al. (1963) reported that pretreatment with phenoxy-

benzamine (50 ug, intra-arterially) increased the effluent noradrenaline from perfused cat spleens from about 23% to 52% of the arterial concentration (8 ng/ml). Cocaine caused a further increase, but phenoxybenzamine did not augment the increase due to cocaine pretreatment. The authors concluded from these results that the α adrenergic receptors play no role in the inactivation of noradrenaline. However, the increase in output of noradrenaline produced by phenoxybenzamine in these experiments was much less than that reported by Brown and coworkers (1957, 1960, 1961) and by Kirpekar and Cervoni (1963) after splenic nerve stimulation. This difference may have been due to the fact that Thoenen et al. studied only venous samples taken during the first 10 minutes after phenoxybenzamine administration, when the action of this agent probably was still incomplete.

In contrast to the results of Thoenen et al., Kirpekar and Cervoni (1963) found that the α adrenergic blocking agents phenoxybenzamine and phentolamine increased the venous output of noradrenaline from the spleen in response to sympathetic nerve stimulation more than did cocaine, and concluded that "whatever sites cocaine acts on must play only a minor role in the removal of transmitter in spleen". These results, coupled with the finding that phentolamine does not block the uptake of tritiated noradrenaline in the spleen (Hertting et al., 1961b), led Kirpekar and Cervoni to state that receptors on effector cells play a major role in terminating the action of the sympathetic mediator.

Gillespie and Kirpekar (1965) very recently restudied the effects of α adrenergic blocking agents on the disposition of catecholamines in the spleen, using a constant slow infusion of noradrenaline

(0.625 ug/min.) rather than nerve stimulation. Their average control recovery in the venous effluent (29%) was increased to 80% by both cocaine and phenoxybenzamine, and to about 60% by Hydergine and phentolamine. On the basis of these results they revised the postulated role of adrenergic receptors in amine disposition to that of a "brake", which promotes nerve uptake by delaying diffusion of catecholamine away from the region of the nerve endings. These workers considered that phentolamine and Hydergine prevented this function of the receptors, whereas phenoxybenzamine blocked both the tissue receptors and the uptake by nerves. It is difficult, to reconcile the experimental observations in this report with the derived theory. Although the proposed "brake" might function in relation to noradrenaline released by nerves, it would seem to have no role under the conditions of their experiments, which provided a constant gradient of amine from the bloodstream toward the uptake site, presumably nerves.

Other investigators have attempted to take advantage of the selectivity of α and β adrenergic blocking agents in assessing the mechanism by which they alter catecholamine disposition. Farrant et al. (1964) studied the effects of phenoxybenzamine (POB) and dichloroisoproterenol (DCI) on the uptake of unlabelled noradrenaline by a variety of cat and rat tissues. POB inhibited uptake by cat kidney but not uterus, whereas DCI prevented uptake by uterus but not by kidney. Thus, uptake was prevented by the agent capable of blocking the dominant adrenergic receptors in each organ. However, POB also blocked uptake by rat spleen, heart and uterus, but potentiated, rather than blocked, responses of the heart and uterus to noradrenaline. These workers concluded that both

POB and DCI can inhibit the uptake and storage of noradrenaline, by an action independent of the blockade of adrenergic receptors. However, their results did not include any instance in which receptors but not tissue uptake of noradrenaline was inhibited.

The contribution of adrenergic receptors to the disposition of the sympathetic mediator and the mechanism by which adrenergic blocking agents inhibit the inactivation of catecholamines are still quite unclear. Phenoxybenzamine and some other blocking agents appear to have an action unrelated to occupancy of tissue receptors. Whether this is a blockade of uptake by nerves appears to be still unsettled, although Malmfors (1965) recently reported fluorescence microscopic observations indicating that any effect of POB on tissue uptake is not predominantly at nerve cell membranes. He found that this agent did not inhibit the accumulation of catecholamine by nerves in the rat iris, whereas cocaine was highly effective. However, the almost complete lack of evidence, except limited observations which did not appear entirely explicable on the basis of an action limited to α receptors, that phenoxybenzamine acts through inhibition of a transport process at the nerve cell membrane, as postulated for cocaine, did not prevent rapid acceptance of this hypothesis, and it remains to be seen whether Malmfors' observations will shake the confidence currently placed in it. Although the reported observations are not entirely consistent, it seems reasonably clear that phenoxybenzamine and, probably, other adrenergic blocking agents cause a greater increase in the outflow of noradrenaline after nerve stimulation than does cocaine. This suggests that if there is some common mechanism of action, the former have an additional action not shared by cocaine.

V. RESULTS OF STUDIES ON THE DISPOSITION OF SYMPATHOMIMETIC AMINES

A. PHENYLEPHRINE (3×10^{-8}) CONTRACTED AORTIC STRIPS

1. Relaxation in Oil:

A concentration of phenylephrine was selected which consistently produced contractions of somewhat less than half maximal amplitude. Phenylephrine (1×10^{-8}) produced contractions with a mean height of 12.5 mm in 24 aortic strips, but the range was from 4 to 15 mm. Responses in the lower part of this range would not have been suitable for studies of the course of subsequent relaxation. The concentration chosen as a standard was 3×10^{-8}, which produced a mean height of contraction of 26.3 mm before oil immersion in 6 control strips; none responded with a contraction height of less than 23 mm. Only 2 of a total of 24 strips with various pretreatments responded to this concentration of phenylephrine with sustained contractions (plateau height) of less than 20 mm.

Four strips were cut from each aorta and randomly assigned to the 4 treatment groups used in the investigation of the inactivation of phenylephrine. In any given experiment, the time of addition of phenylephrine and the time of oil immersion were the same for all strips.

a. Control strips: Phenylephrine (3×10^{-8}) was added to the muscle chambers and they were drained and filled with oil 20 to 30 minutes later. The mean height of contraction of 6 strips before oil immersion was 26.3 mm.

b. Cocainized strips: Phenylephrine (3×10^{-8}) was added to the muscle chambers and after the contractions had reached a plateau, usually by about 10 minutes, cocaine was added either in logarithmic increments from 1×10^{-7} to 1×10^{-5} or in one concentration of 1×10^{-5}.

Cocaine (1×10^{-5}) was chosen as the final concentration because it usually produced maximal potentiation of phenylephrine contractions. Higher concentrations produced less potentiation, or even a decrease in the amplitude of contraction. Ten to 20 minutes after the addition of cocaine the chambers were drained and filled with oil. The mean height of contraction of 6 strips before oil immersion was 29.2 mm.

c. Iproniazid pretreated strips: Strips were pretreated with iproniazid as described in Methods and then treated in the same way as the controls. The mean height of contraction of 6 strips before oil immersion was 28.5 mm.

d. Iproniazid pretreated, cocainized strips: Strips were pretreated with iproniazid as described in Methods and then with cocaine as described for the strips of group b, above. The mean height of contraction of 6 strips before oil immersion was 30.0 mm.

The records from a typical experiment are shown in figure 12 and the combined results from 6 complete experiments in figure 13. Control strips relaxed 38.8% in 5, 73.0% in 15 and 84.0% in 30 minutes after oil immersion. Strips treated with cocaine relaxed at a significantly slower rate than did the control strips, only 20.3% in 5, 50.7% in 15 and 71.7% in 30 minutes. Iproniazid pretreated strips responded to oil immersion with an initial abrupt decrease in contraction height, followed by very slow further relaxation. A similar rapid initial relaxation at oil immersion was noted in strips contracted by various agonists after blockade of major inactivation mechanisms for the drug involved. There is at present no adequate explanation for this phenomenon, but it may reflect some type of drug redistribution. Iproniazid
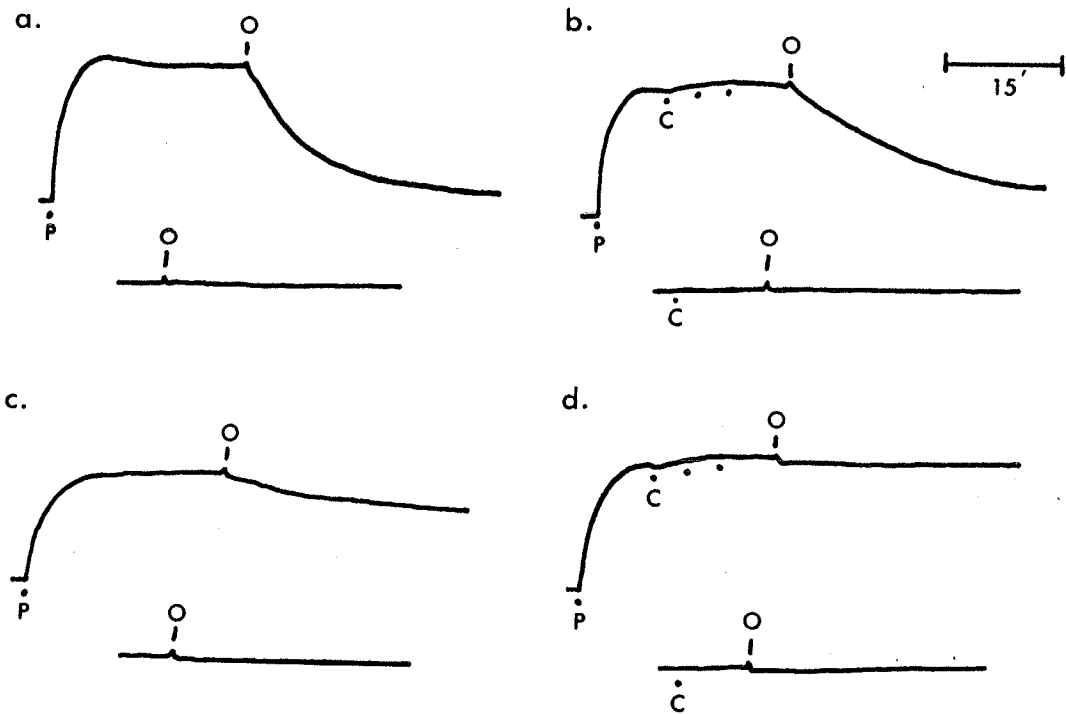
Fig. 12. Effects of Cocaine and Iproniazid on Relaxation of Phenyl-
ephrine Contracted Aortic Strips after Oil Immersion.

Upper record of each pair is the response of a strip contracted
with phenylephrine (P) (3×10^{-8}) and subsequently allowed to relax in
oil (O). a. Control strip. b. Strip exposed to cocaine (C) in cumula-
tive concentrations of 1×10^{-7}, 1×10^{-6} and 1×10^{-5} (dots). c. Strip
pretreated with iproniazid. d. Strip pretreated with iproniazid and ex-
posed to cocaine as in b. Lower record of each pair is of a strip
treated with the same agents except for phenylephrine; cocaine was added
in a single concentration of 1×10^{-5}. All strips were from the same
aorta.
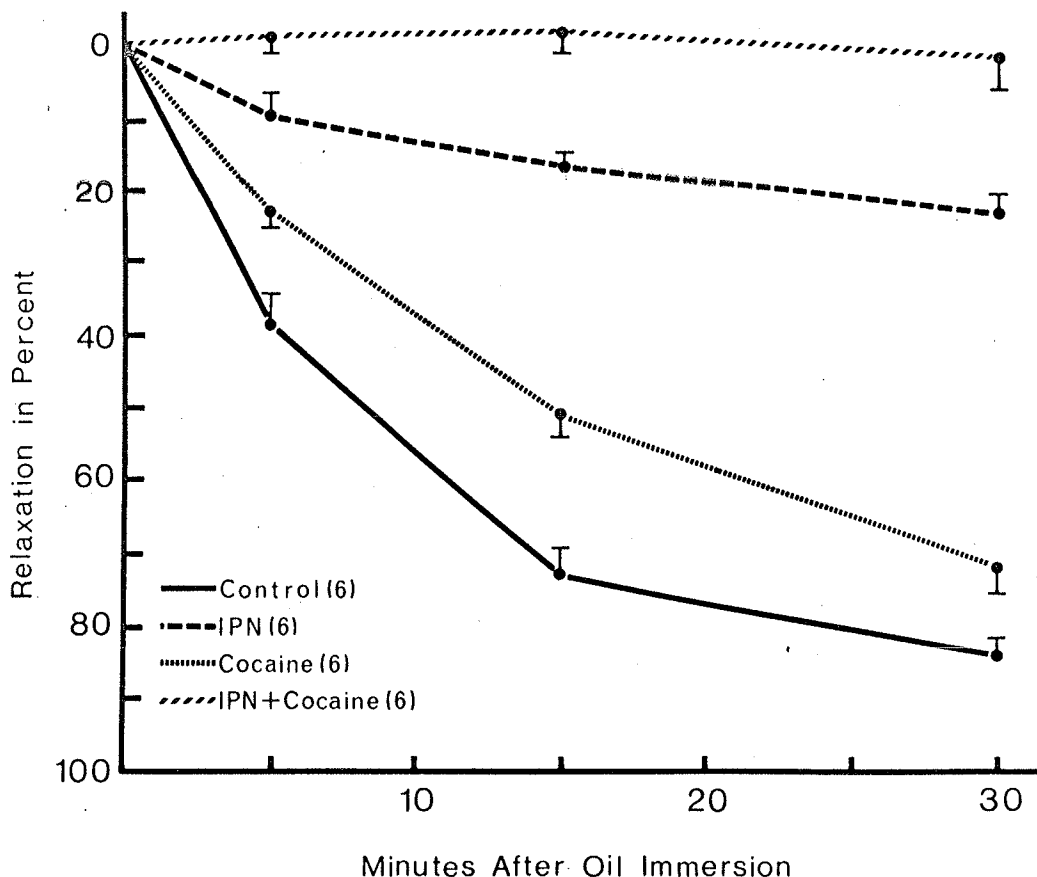
Fig. 13.   Effects of Cocaine and Iproniazid on the Relaxation of Phenyl-ephrine Contracted Aortic Strips after Oil Immersion.

Each curve shows the mean of 6 strips, one in each group from each of 6 aortas, contracted by phenylephrine (3×10^{-8}) and allowed to relax in oil.   Bars indicate standard errors of means.   See text for de-tailed description of experiments.

Statistical Analysis:

	Min.	
Cocaine vs. Control	5	P < 0.01
	15	P < 0.01
	30	P < 0.02
Iproniazid vs. Cocaine	5	P < 0.02
	15	P < 0.001
	30	P < 0.001
Iproniazid + Cocaine vs. Iproniazid	5	P < 0.02
	15	P < 0.001
	30	P < 0.01

pretreated strips relaxed only 16.0% in 15 and 23.0% in 30 minutes. Cocaine increased the time required for 50% relaxation to about 1.8 times, and iproniazid the time required for 20% relaxation (50% was not reached during the observation period) to about 9 times that of the controls.

Cocaine significantly slowed the rate of relaxation of both iproniazid pretreated and control strips, and a combination of the MAO inhibitor and cocaine almost completely prevented the relaxation of phenylephrine contracted strips after oil immersion, a mean of only 1.3% in 30 minutes. On the basis of experiments showing that relaxation is a function of the residual concentration of agonist in the tissue (Section III, A), it appears that treatment with both iproniazid and cocaine essentially completely abolished the ability of the aortic strip to inactivate phenylephrine. Neither cocaine, iproniazid nor the combination affected the basal tone of the strips.

The relative contributions of the cocaine sensitive mechanism and of MAO to the inactivation of higher concentrations of phenylephrine were not studied in detail. However, one experiment performed with a concentration of 1×10^{-6} phenylephrine indicated that deamination was the only significant route of inactivation (Fig. 14). The control strip relaxed 96.2% and the iproniazid pretreated strip only 14.5% in 55 minutes after oil immersion.

As shown earlier (Section III, B, 1, f), iproniazid does not alter the rate of relaxation in oil of aortic strips contracted by Cobefrine, a sympathomimetic amine which is not a substrate for MAO. Three strips contracted with Cobefrine before and after exposure to
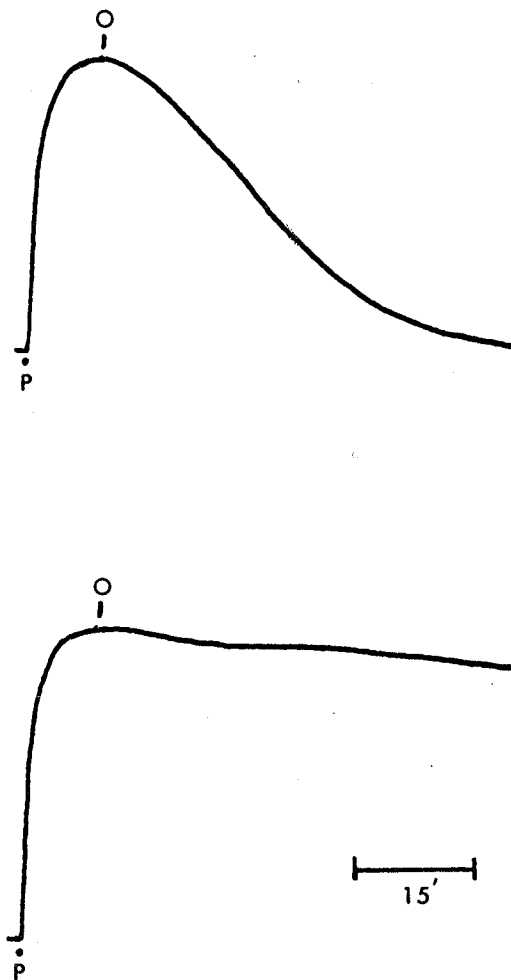
Fig. 14.   Effect of Iproniazid on Relaxation after Oil Immersion of Aortic Strips Contracted by a High Concentration of Phenylephrine.

Upper record shows response of a control strip contracted with phenylephrine (P) (1 X 10^{-6}), and lower record the response of a strip pretreated with iproniazid.   Oil immersion indicated by (O).

iproniazid relaxed 68.5% and 69.1% in 5 minutes, respectively. This provided convincing evidence that the effect of iproniazid on the rate of relaxation of strips contracted by phenylephrine was due to inhibition of MAO.

To further rule out participation of actions other than inhibition of MAO in the observed effects of iproniazid, its effect on the relaxation of aortic strips contracted by phenylephrine was compared with that of isoniazid, a congener which shares the reactive hydrazine moiety but does not inhibit MAO. Three strips were contracted with phenylephrine (3×10^{-8}) and allowed to relax in oil. They subsequently were treated with isoniazid (2×10^{-4}), as described in Methods for iproniazid, and again contracted with phenylephrine. Isoniazid did not alter the rate of relaxation in oil, which was 55.8% in 5 minutes before and 56.0% after treatment (Table VIb).

2. Effect of a Higher Concentration of Iproniazid:

It has been reported, on the basis of manometric determinations, that MAO in aortic strips is completely and irreversibly inhibited by a 30 minute exposure to a concentration of 1×10^{-4} iproniazid (Furchgott, 1955). A somewhat higher concentration (2×10^{-4}) was used in most of the experiments reported in this thesis. In addition, the completeness of enzyme inhibition by this concentration was checked by comparing the rates of relaxation in oil of strips contracted by phenylephrine (3×10^{-8}) after pretreatment with iproniazid 2×10^{-4} or 1×10^{-3}. The 5 times greater concentration of inhibitor did not slow relaxation more than did the standard. Two strips treated with 2×10^{-4} and 2 with 1×10^{-3} iproniazid relaxed 30.0% and 32.2%, respectively,

TABLE VI

EFFECTS OF IPRONIAZID AND ISONIAZID ON THE RELAXATION

OF PHENYLEPHRINE CONTRACTED STRIPS IN OIL

(a) Effects of Concentrations of Iproniazid on the Relaxation of Phenylephrine (3×10^{-8}) Contracted Aortic Strips

Iproniazid Conc.	Time after Oil Immersion (min.)		
	5	15	30
(2×10^{-4}) (2)	10.2%	17.1%	30.0%
(1×10^{-3}) (2)	12.8%	22.5%	32.2%

(b) Effects of Isoniazid on the Relaxation of Phenylephrine (3×10^{-8}) Contracted Aortic Strips

Isoniazid Conc.	Time after Oil Immersion (min.)		
	5	15	30
O (3)	55.8%	84.3%	92.1%
(2×10^{-4}) (3)	56.0%	84.3%	91.6%

Figures in parentheses refer to number of strips from which means were determined. In the isoniazid series, each strip served as its own control.

in 30 minutes (Table VIa). Since iproniazid is a nonequilibrium

inhibitor of MAO, even large increases in substrate concentration would

not reduce the degree of enzyme inhibition produced.

3. Effects of JB-516 (Catron):

In some experiments the MAO inhibitor JB-516 (Catron) was

used in place of iproniazid, in an attempt to provide further evidence

that enzyme inhibition per se was responsible for the observed effects

of the latter. JB-516 is a hydrazine derivative which is a considerably

more potent inhibitor of MAO than is iproniazid. In a concentration

of 1×10^{-5} it completely eliminated relaxation in oil over a 30 minute

period an effect similar to that of iproniazid and cocaine combined.

JB-516 was therefore studied for a possible cocaine-like action. In 2

experiments cocaine (1×10^{-5}) failed to potentiate responses of JB-516

pretreated strips to phenylephrine, and in one, it produced a slight

depression, similar to that seen when strips already treated with this

concentration of cocaine are exposed to one 10 times higher. This

effect and the potentiation by cocaine (1×10^{-5}) of the phenylephrine-

induced contraction of a strip cut from the same aorta, but not exposed

to JB-516, are shown in figure 15. JB-516 at a concentration of

1×10^{-6} only partially inhibited the relaxation of phenylephrine

contracted strips in oil.

The mean height of contraction of 3 strips from 2 aortas

pretreated with JB-516 (1×10^{-5}) in response to phenylephrine

(3×10^{-8}) was 55 mm, and that of 2 control strips from the same aortas,

41.5 mm. It is shown below that iproniazid does not appreciably augment

contractions produced by phenylephrine, and the increase due to JB-516

a.

b.
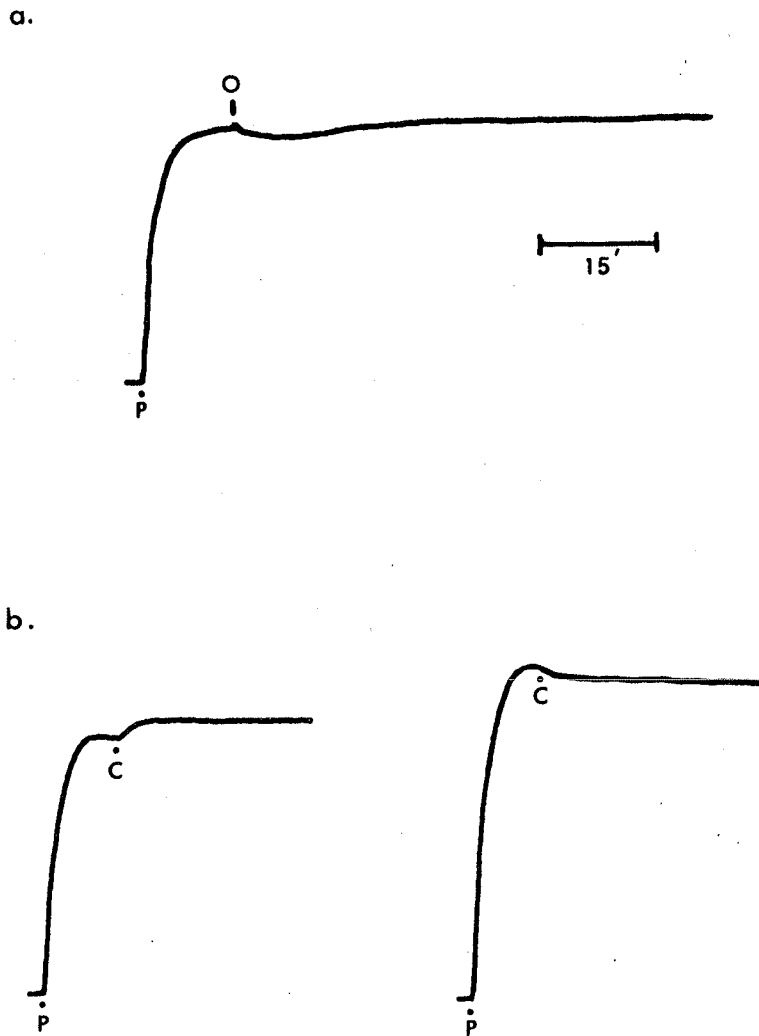
Fig. 15.   Effect of JB-516 on Responses of Phenylephrine Contracted
Aortic Strips.

a.   Behavior in oil (O) of a JB-516 (1 X 10^{-5}) pretreated
strip, from a reserpinized rabbit, contracted with phenylephrine (P)
(3 X 10^{-8}).   b.   Potentiation of the response to phenylephrine
(3 X 10^{-8}) by cocaine (C) (1 X 10^{-5}) (left) and absence of potentiation
by cocaine of the response to phenylephrine of a JB-516 (1 X 10^{-5}) pre-
treated strip from the same aorta.

is further evidence that it has an action unrelated to MAO inhibition.

4. <u>Effect of Iproniazid on Contraction Amplitude:</u>

Since the major tissue mechanism for the inactivation of
phenylephrine was found to be deamination, experiments were designed
to determine if inhibition of MAO potentiated the contractile response
to this amine. The mean peak contraction in response to phenylephrine
(3×10^{-8}) of all strips not exposed to a MAO inhibitor was 27.2 ± 1.5
mm, and that of the enzyme inhibited group 28.4 ± 2.7 mm. This dif-
ference was not statistically significant. A more precise measure
of possible potentiation of responses to phenylephrine by inhibition of
MAO was obtained in experiments in which 2 responses of each strip were
compared. Low concentrations of phenylephrine (3×10^{-9} or 1×10^{-8}),
on the steepest part of the dose-response curve were employed to increase
the sensitivity of the test. After the first contraction, half of the
strips were treated with iproniazid, as described in Methods, and half
served as controls. Each was then reexposed to the same concentration
of phenylephrine used to produce the first contraction (Table VII).

All second responses to 3×10^{-9} phenylephrine were less than
the first, an average of 39.4% for 5 iproniazid treated and 53.2% for
5 control strips. The difference between these two groups is not sta-
tistically significant. Second responses of 12 iproniazid treated strips
to 1×10^{-8} phenylephrine averaged 1.8% greater than the first, whereas
those of the control strips to this concentration were reduced 26.2%.
This difference is significant at the 2% level of probability. It appears
that iproniazid can potentiate responses to at least some concentrations
of phenylephrine, but that this effect is so small that it is not easily
detected.

TABLE VII

EFFECT OF IPRONIAZID ON CONTRACTIONS PRODUCED

BY PHENYLEPHRINE AND COBEFRINE

Phenylephrine

Agonist Conc.	First Contraction (mm)	Interval Treatment	Second Contraction (mm)	Change %
3×10^{-9}	5.6 (1 - 12)	--	2.4 (0 - 5)	- 53.2 $\pm$ 15.9 (5)
	4.8 (1 - 10)	IPN	2.6 (1 - 7)	- 39.4 $\pm$ 11.5 (5)
				N.S.
1×10^{-8}	13.0 (6 - 10)	--	9.5 (3 - 15)	- 26.2 $\pm$ 7.2 (12)
	12.0 (4 - 15)	IPN	12.1 (4 - 18)	+ 1.8 $\pm$ 8.5 (12)
				P = <0.02, >0.01

Cobefrine

1×10^{-8}	2.0 (1 - 3)	--	2.0 (1 - 3)	0 (3)
	2.3 (1 - 3)	IPN	2.3 (1 - 3)	0 (3)

Figures in parentheses on the extreme right indicate number of
strips from which means were determined. Those to right of con-
traction amplitudes indicate the range of values. Changes in
response were determined on the basis of intraindividual comparison.

5. <u>Effect of Iproniazid on Cobefrine-Induced Contractions</u>:

Studies of the relaxation of phenylephrine contracted aortic strips in oil indicated that MAO was the major mechanism for the inactivation of this amine, but pretreatment with iproniazid produced little potentiation of responses to it. Kamijo <u>et</u> <u>al</u>. (1956) reported that high concentrations of iproniazid reversibly blocked α adrenergic receptors, and although the concentrations used here were lower than those employed by Kamijo and coworkers, and any blockade produced should have been reversed by the washing to which the strips were subjected, it appeared possible that some potentiation was masked by α receptor blockade. This possibility was investigated in studies on a sympathomimetic amine which is not a substrate for MAO.

Just threshold contractions were produced in 6 strips by Cobefrine (1×10^{-8}). After washout and recovery of basal tone, 3 strips were treated with iproniazid, and 3 served as controls. Second responses of the strips in both groups to the same concentration of Cobefrine were the same as the first (Table VII), indicating that the iproniazid had produced no detectable α adrenergic blockade.

6. <u>Effects of Iproniazid on Tyramine- and Dopamine-Induced Contractions</u>:

Evidence that iproniazid pretreatment can markedly potentiate responses to some amines which are substrates for MAO is presented in figure 16. Dose-response curves for tyramine were obtained on 12 aortic strips from 5 animals. Six of the strips were pretreated with iproniazid and 6 served as controls. Because of marked variability in the responses of strips from different animals to tyramine, apparently related, in part, to seasonal variations, all of these experiments were performed
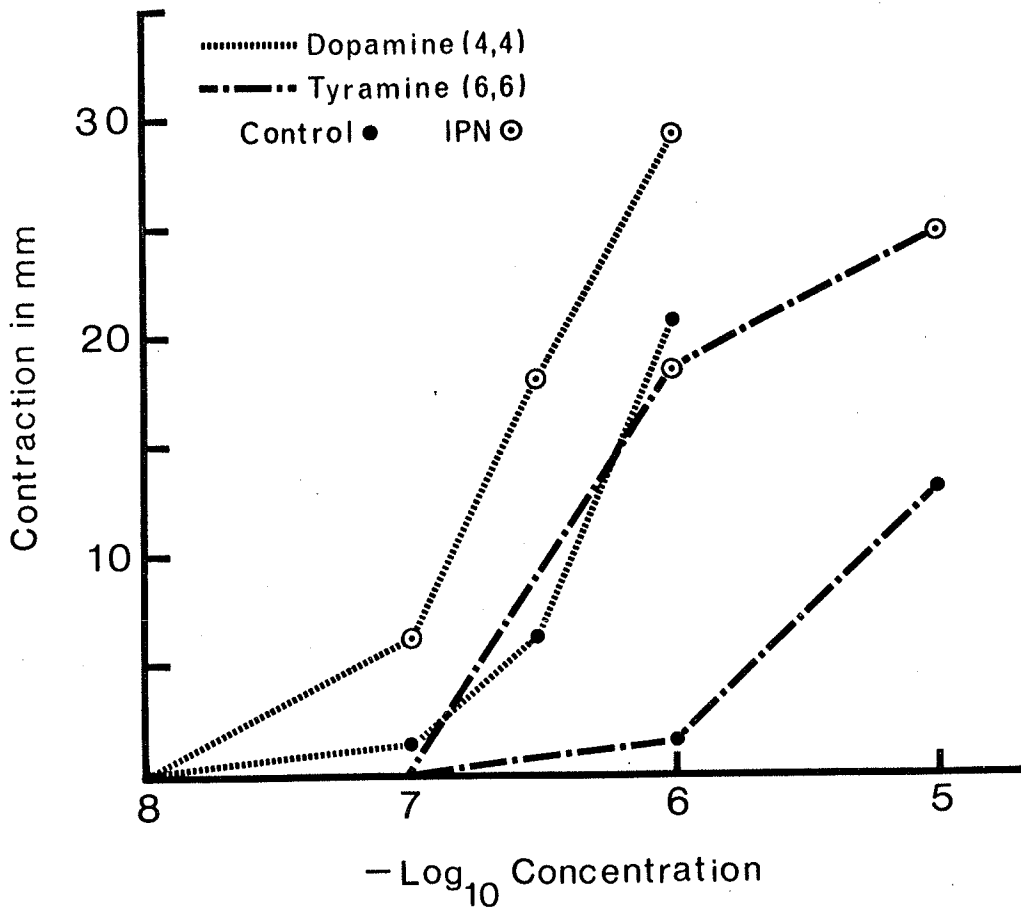
Fig. 16.   Effect of Iproniazid (IPN) Pretreatment on Tyramine and
Dopamine Log Dose-Response Curves.

Figures in parentheses indicate the number of complete
experiments on which each curve is based. Bars indicate standard errors
of means.

within a 10 day period and each strip pretreated with the MAO inhibitor

was matched with a control strip from the same animal. Inhibition of

MAO produced about a 20 fold shift to the left of the tyramine dose-

response curve. A similar shift was reported by Furchgott et al. (1963).

Although tyramine thresholds varied markedly in strips from different

aortas, the degree of potentiation by iproniazid remained relatively

constant.

Although dopamine is a substrate for catechol-O-methyl

transferase (COMT) as well as for MAO, earlier observations (Section III,

B, 1, f) indicated that responses to it are potentiated by inhibition

of MAO. Responses of 4 control strips to cumulative concentrations of

dopamine were compared with those of 4 iproniazid pretreated strips

from the same aortas (Fig. 16), and it was found that dopamine dose-

response curve was shifted to the left about 3 fold. These experiments

were performed on strips from reserpinized animals to eliminate possible

effects of catecholamines released from endogenous stores.

7. Effect of Cocaine on Contraction Amplitude:

Cocaine (1×10^{-5}) was added to the muscle chambers after the

responses of aortic strips to phenylephrine had reached a plateau, and

the augmentation due to the cocaine calculated as a percent of the

contraction height immediately before its addition. Ten control strips,

contracted with phenylephrine (3×10^{-8}), were potentiated a mean of

3.1 mm (11.7%) and 6 iproniazid pretreated strips, 3.0 mm (11.2%).

Thus, inhibition of MAO did not alter the potentiating action of

cocaine. The contraction height after the addition of cocaine was

converted to equivalent concentration of phenylephrine alone required

to produce a contraction of the same amplitude from a standard phenylephrine dose-response curve (Fig. 1). In terms of equivalent phenylephrine concentration, cocaine (1×10^{-5}), produced a potentiation of about 100%. Cocaine alone had no effect on the basal tone of aortic strips, as has been previously reported (Furchgott et al., 1963; Maxwell et al., 1962).

8. Effects of Methylphenidate:

To confirm that the delay in the relaxation of phenylephrine contracted aortic strips in oil produced by cocaine was an expression of the same action observed as a delayed inactivation of sympathomimetic amines in vivo, effects of cocaine and methylphenidate were compared. This drug has been reported to have the same spectrum of effects on adrenergic mechanisms as does cocaine (Furchgott, 1960b), and both contain a methylated β-alanine moiety.

Cumulative addition of methylphenidate to chambers containing phenylephrine (3×10^{-8}) contracted strips which had reached a plateau showed that the maximal increment in contraction amplitude was produced by a concentration of 1×10^{-6} (Fig. 17), equivalent to that produced by cocaine (1×10^{-5}). This indicated that the potency of methylphenidate is about 10 times that of cocaine in potentiating responses to phenylephrine. Also, methylphenidate (1×10^{-5}), added to a concentration of 1×10^{-6}, depressed the amplitude of phenylephrine-induced contractions (Fig. 17b), an effect very similar to that of adding cocaine (1×10^{-4}) in the presence of cocaine (1×10^{-5}). Evidence that the mechanism by which methylphenidate and cocaine potentiate phenylephrine responses is the same, is presented in figure 17c.
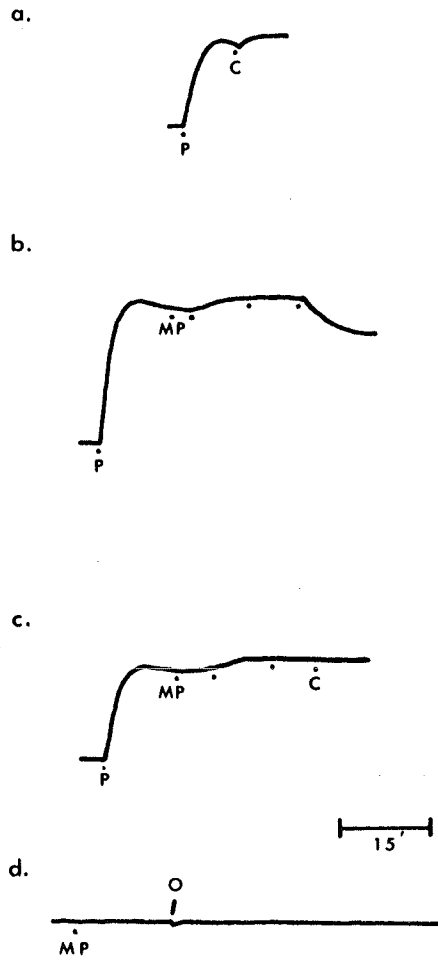
Fig. 17.   Interactions of Cocaine and Methylphenidate on Aortic Strips.

a.   Response to cocaine (C) (1 X 10^{-5}) of a strip contracted by phenylephrine (P) (3 X 10^{-8}).   b. Response to cumulative concentrations of methylphenidate (MP) (1 X 10^{-8}, 1 X 10^{-7}, 1 X 10^{-6} and 1 X 10^{-5}) of a strip contracted by phenylephrine.   c. Absence of potentiation by cocaine of the response of a strip to phenylephrine in the presence of methyl-phenidate (1 X 10^{-8}, 1 X 10^{-7} and 1 X 10^{-6}).   d. Lack of effect of methyl-phenidate alone (1 X 10^{-6}) on the basal tone of a strip in Krebs solution or after oil immersion (O).   All strips were from the same aorta.

Cocaine caused no potentiation in addition to that already produced by methylphenidate (1×10^{-6}). This is in agreement with the observations of Maxell et al. (1962).

Contractions were produced by phenylephrine (3×10^{-8}) in 10 strips from 4 aortas. These were then exposed to methylphenidate (1×10^{-6}), cocaine (1×10^{-5}), or both, added either cumulatively or as a single concentration, and their subsequent relaxation in oil recorded (Fig. 18). Methylphenidate treated strips relaxed 26.7% and cocaine treated strips 28.0% in 5 minutes, compared to 50.8% for the controls. The combination of cocaine and methylphenidate slowed relaxation no more than did either alone. Methylphenidate alone had no effect on the basal tone of aortic strips in either Krebs solution or oil.

9. Relaxation in Air:

Six aortic strips were contracted with phenylephrine (1×10^{-8}) and the muscle chambers then drained and the tissues left exposed to air. Five minutes after draining the baths, the strips were washed once quickly with Krebs solution and their relaxation in air recorded for an additional 5 minutes. Two control strips relaxed $45.3 \pm 0.9\%$, 2 cocaine (1×10^{-5}) treated strips $32.5 \pm 0.9\%$ and 2 iproniazid pretreated strips $18.7 \pm 4.4\%$ in 5 minutes (Fig. 19). The rates of relaxation of both the cocainized and iproniazid pretreated strips was significantly less than that of the controls at the 5% level of probability.

Although the concentration of phenylephrine used in this experiment was 1×10^{-8}, rather than 3×10^{-8}, as in the oil immersion
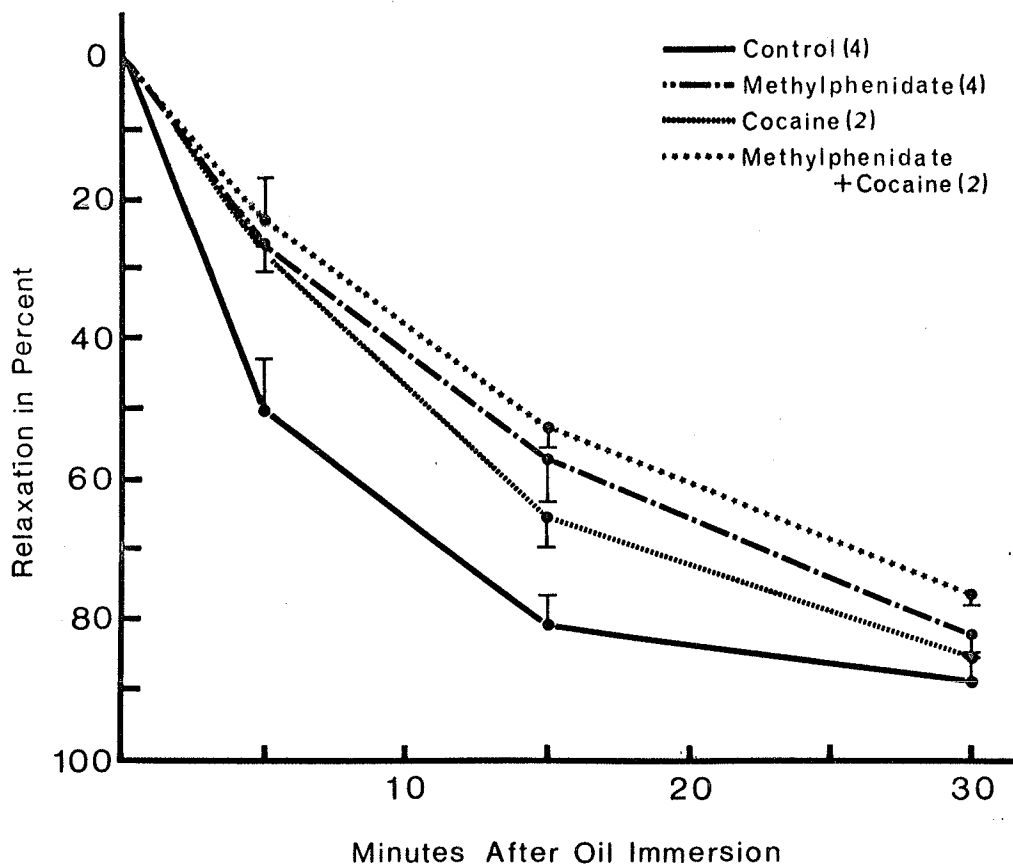
Fig. 18. Effects of Methylphenidate and Cocaine on Relaxation of Phenylephrine Contracted Aortic strips after Oil Immersion.

Concentrations used were: Phenylephrine - 3×10^{-8}, methylphenidate - 1×10^{-6}, cocaine - 1×10^{-5}. Figures in parentheses indicate number of preparations represented by each curve. Bars indicate standard errors. Rate of relaxation of strips treated with methylphenidate was significantly slower than that of the control. ($P < 0.05$ at both 5 and 15 min.).
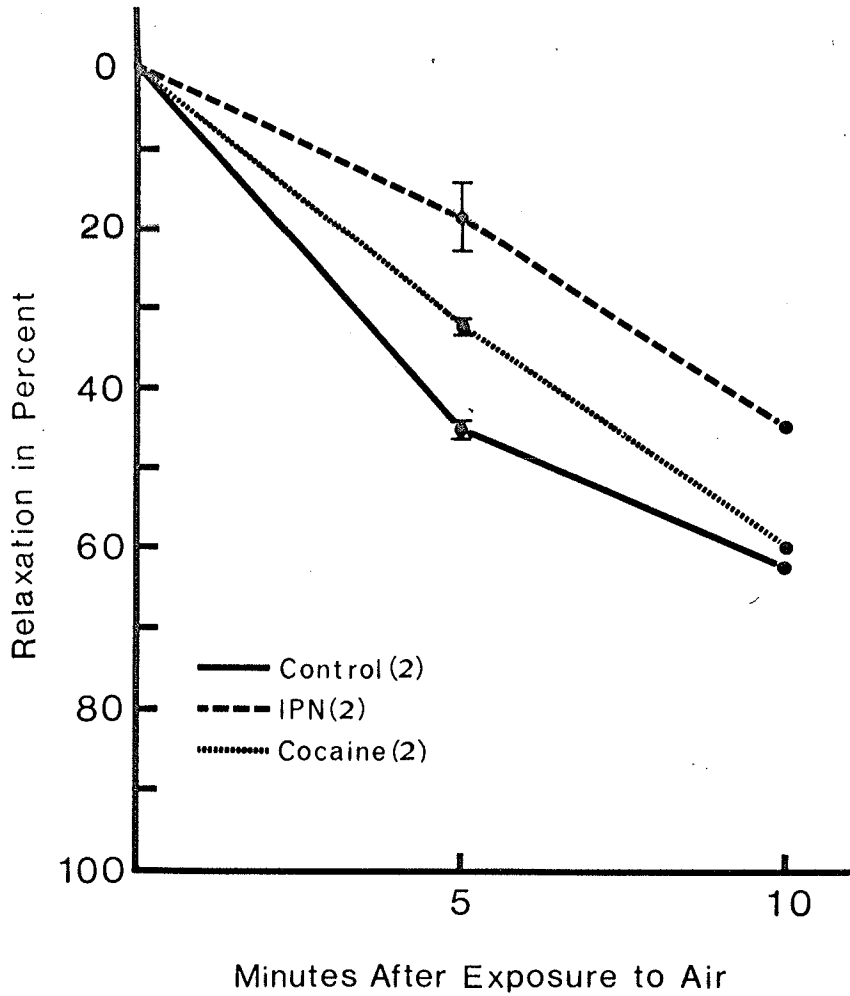
Fig. 19.   Effects of Cocaine and Iproniazid on Relaxation of Phenyl-
ephrine Contracted Aortic Strips in Air.

Strips were contracted by phenylephrine (1×10^{-8}) and treated
with iproniazid (IPN) or cocaine (1×10^{-5}) as in oil immersion studies.
The muscle chambers were drained and the strips left exposed to air
except for a single quick wash with Krebs solution at 5 minutes.   Figures
in parentheses indicate number of preparations represented by each curve.
Bars indicate standard errors of means.

Statistical Analysis (at 5 min.):

Control vs. Cocaine	$P < 0.02$
Control vs. Iproniazid	$P < 0.05$
Iproniazid vs. Cocaine	$P < 0.1$

studies, the results show the same relative importance of MAO and of the cocaine sensitive mechanism, in the inactivation of phenylephrine.

10. Comment:

Phenylephrine was chosen as the first sympathomimetic amine to be subjected to detailed study by the oil immersion technique because the mechanisms for its biological inactivation appeared to be less complex than those for the catecholamines. Results reported by other investigators, reviewed in the Historical Introduction, indicated that its inactivation in tissues would involve enzymatic deamination and a mechanism sensitive to cocaine. The structure of phenylephrine and in vitro experiments with liver homogenates (Blaschko et al., 1937b) indicate that it is a good substrate for MAO. Accumulated data on the action of cocaine indicate that it can impair the inactivation of many sympathomimetic amines by binding. Thus, the results obtained were, at least qualitatively, not surprising. However, the finding that the combination of a MAO inhibitor and cocaine almost completely eliminated the ability of aortic strips to inactivate phenylephrine over a period of at least 30 minutes demonstrated that no other mechanisms contribute appreciably to the inactivation of this amine in aortic tissue. This could not have been easily demonstrated by other techniques, where exchange of drug with the bathing medium or blood makes it exceedingly difficult to account quantitatively for the total amount of any drug in a given tissue.

The various experiments described support the validity of using inhibitors of specific metabolic pathways in conjunction with oil immersion in assessing routes of drug inactivation in tissue. The qualitative similarity of the relaxation of aortic strips treated with

various inhibitors in air and oil provides additional evidence that true effects on rates of inactivation are being recorded.

Attempts to investigate the pathways of endogenous inactivation of noradrenaline and adrenaline with the technique of oil immersion will be described in the following sections.

B. NORADRENALINE (1×10^{-8}) CONTRACTED AORTIC STRIPS

1. Relaxation in Oil:

A concentration of 1×10^{-8} noradrenaline was selected for study with the oil immersion technique because it produced contractions of about half maximal amplitude, comparable to those produced by phenylephrine (3×10^{-8}), a mean of 27.0 mm before oil immersion in 10 control strips. Only one control strip responded to this concentration of noradrenaline with a contraction height of less than 21 mm, and only 6 of 76 strips, subjected to various pretreatments, responded with contractions of less than 21 mm before oil immersion.

Eight treatment conditions were employed to investigate mechanisms for the inactivation of noradrenaline. In a majority of experiments strips cut from the same aorta were utilized for all 8 treatments, to reduce variability. Mean relaxation curves for all groups are shown in figure 20a and b, and typical traces obtained with several of the treatment conditions are shown in figure 21. To compare accurately the rates of relaxation in oil, the time required for each strip to relax 50% was directly measured and mean time calculated for each treatment condition. Under circumstances where 50% relaxation was

Fig. 20, a and b.   Effects of Iproniazid, Tropolone and Cocaine on the Relaxation of Noradrenaline Contracted Aortic Strips after Oil Immersion.

Treatment with iproniazid (IPN) and tropolone (Trop) was as described in Methods. Concentration of noradrenaline was 1×10^{-8} in all experiments, and that of cocaine 1×10^{-5}, except where specifically indicated otherwise. Figures in parentheses indicate the number of preparations represented by each curve. Bars represent standard errors of means. See text for detailed description of experiments.

Statistical analysis:

	Min.
Control vs. Cocaine	5 – $P < 0.05$
	15 – $P < 0.2$
Control vs. Iproniazid	N.S.
Control vs. Tropolone	5 – $P < 0.05$
	15 – $P < 0.1$
Iproniazid + Cocaine vs. Cocaine	N.S.
Iproniazid + Cocaine vs. Control	5 – $P < 0.02$
	15 – $P < 0.1$
Tropolone vs. Cocaine + Tropolone	5 – $P < 0.001$
	15 – $P < 0.001$
	30 – $P < 0.01$
Tropolone vs. Iproniazid + Tropolone	5 – $P < 0.001$
	15 – $P < 0.001$
	30 – $P < 0.001$
Iproniazid + Tropolone vs. Iproniazid + Tropolone + Cocaine	5 – $P < 0.01$
	15 – $P < 0.001$
	30 – $P < 0.01$
Iproniazid + Tropolone + Cocaine (1×10^{-5}) vs. Iproniazid + Tropolone + Cocaine (1×10^{-4})	30 – $P < 0.2$

a.

Control (10) ----IPN (10)
............Cocaine (9) —·—Trop (6)
^^^^^IPN+Trop+Cocaine (9)
IPN+Trop+Cocaine 1X10^{-4} (6)

Relaxation in Percent

0
20
40
60
80
100

10   20   30

Minutes After Oil Immersion

b.

----IPN (10)
—·—Trop (6)
............Cocaine (9)
••••IPN+Trop (7)
////IPN+Cocaine (8)
—··—Trop+Cocaine (5)

Relaxation in Percent

0
20
40
60
80
100

10   20   30

Minutes After Oil Immersion
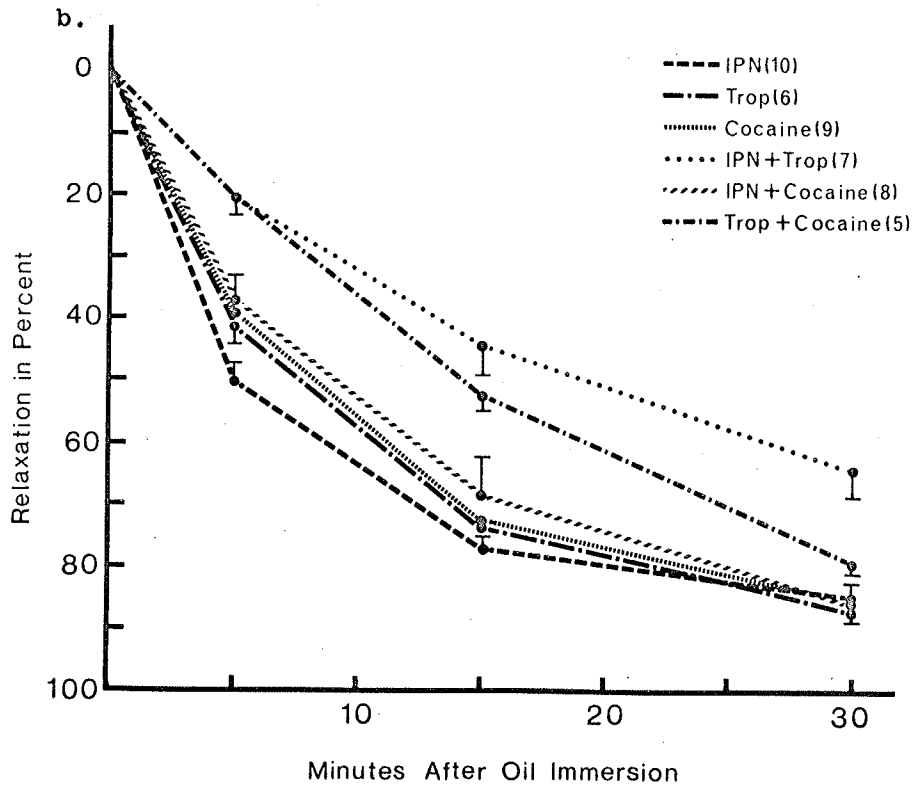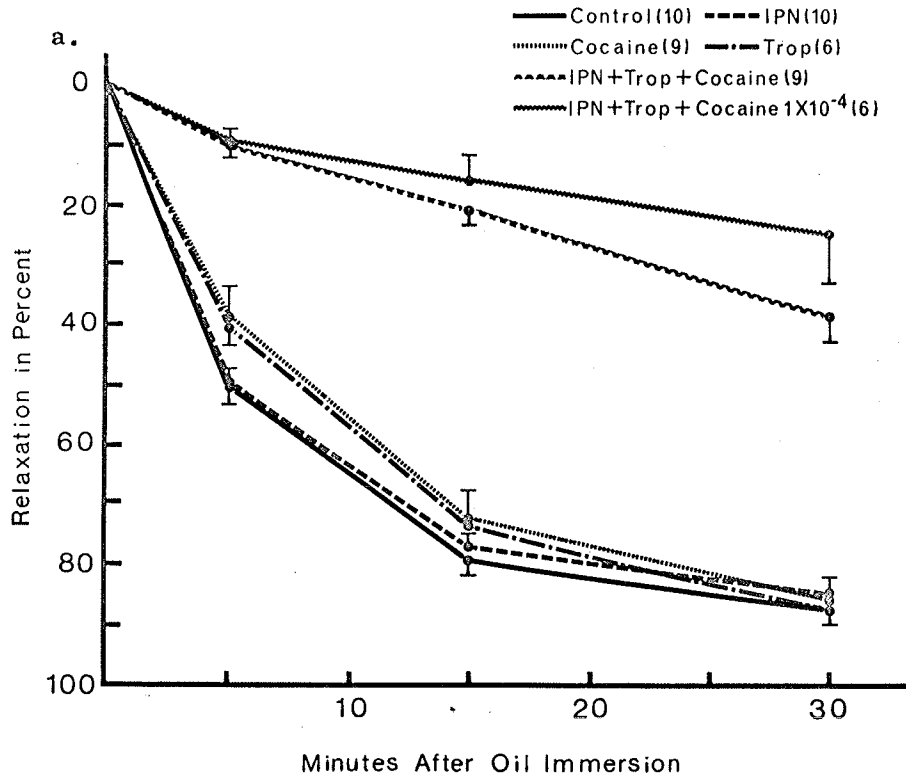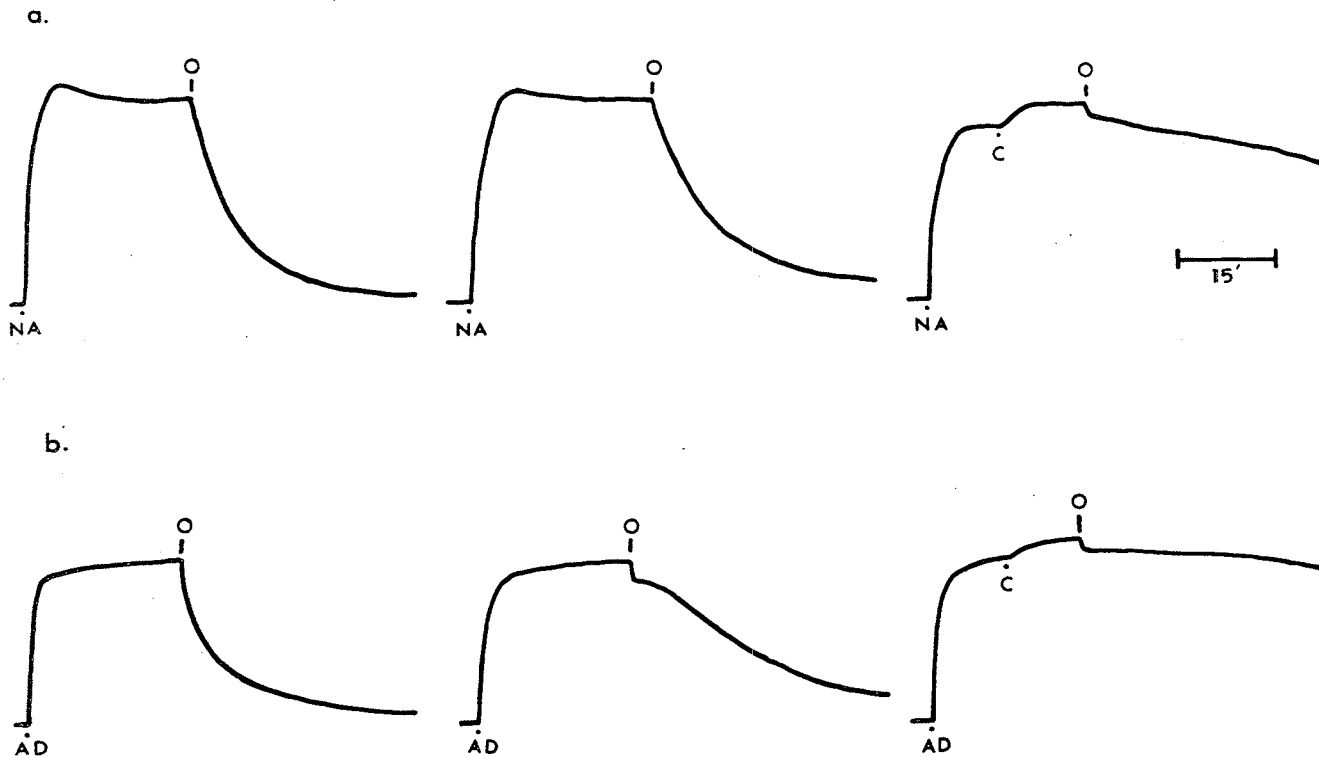
Fig. 21.   Effects of Cocaine and Enzyme Inhibitors on Noradrenaline and Adrenaline
Contracted Aortic Strips.

      a.   Strips contracted by noradrenaline (NA) (1×10^{-8}): left, control strip;
center, strip pretreated with tropolone; right, strip pretreated with iproniazid and
tropolone, and cocaine (C) (1×10^{-5}) added before oil immersion (O).   b.   The same as
a, except that strips were contracted with adrenaline (AD) (1×10^{-8}) instead of nora-
drenaline.

usually not achieved within 30 minutes after oil immersion, comparisons were made at some lesser percent relaxation. These results are summarized in Table VIII.

a. <u>Control strips</u>: Noradrenaline (1×10^{-8}) was added to the muscle chambers and 20 to 30 minutes later they were drained and filled with oil. These strips relaxed in oil faster than did those contracted with phenylephrine. Five minutes after oil immersion they had relaxed a mean of 51.0%, and by 15 minutes relaxation was 79.6% complete.

b. <u>Cocainized strips</u>: Noradrenaline (1×10^{-8}) was added to the chambers and when the amplitude of contraction had reached a plateau, usually after about 10 minutes, cocaine was added either in logarithmic increments from 1×10^{-7} to 1×10^{-5}, or in a single concentration of 1×10^{-5}. A concentration of 1×10^{-5} cocaine was chosen to facilitate comparison with the studies on phenylephrine, in which this concentration was used. Maximal potentiation of responses to noradrenaline was usually achieved with a concentration of 1×10^{-4} cocaine; experiments in which this concentration was used are specifically indicated. Ten to 20 minutes after the addition of cocaine the chambers were drained and filled with oil. The mean height of contraction of 9 strips before oil immersion was 31.1 mm. Cocaine (1×10^{-5}) significantly slowed relaxation, which was only 39.0% complete after 5 minutes. The time for half relaxation of noradrenaline contracted strips treated with cocaine was 1.5 times that of controls. Cocaine increased the time required for half relaxation of phenylephrine contracted strips to almost 1.8

TABLE VIII

RELAXATION OF NORADRENALINE AND ADRENALINE CONTRACTED
AORTIC STRIPS AFTER OIL IMMERSION

Treatment	NORADRENALINE (1×10^{-8})			ADRENALINE (1×10^{-8})		
	No. Exps.	Time to Relax 50% (min.)	Shift from Control Time	No. Exps.	Time to Relax 50% (min.)	Shift from Control Time
Control	10	5.29 ± 0.5 $2.78 \pm 0.2*$		6	4.18 ± 0.5 $1.65 \pm 0.3*$	
Iproniazid	10	5.14 ± 0.3		6	4.28 ± 0.6	1.02
Tropolone	6	7.03 ± 0.7	1.33	7	12.23 ± 1.2	2.93
Cocaine	9	7.86 ± 1.3	1.49	5	6.58 ± 1.4	1.57
Iproniazid + Tropolone	7	17.0 ± 3.1	3.21	7	25.30 ± 3.3	6.05
Iproniazid + Cocaine	8	8.13 ± 1.2	1.54	5	5.30 ± 0.8	1.27
Tropolone + Cocaine	5	13.88 ± 0.7	2.62	6	24.63 ± 3.7	5.89
Iproniazid + Tropolone + Cocaine	9	$23.81 \pm 2.6*$	8.56	5	$26.98 \pm 2.8*$	16.35

Concentration of cocaine was 1×10^{-5} in all experiments.

* Indicates time to relax 30 rather than 50%, compared to control at 30% relaxation.

times that of the controls (from 8.25 to 14.5 minutes). Two strips treated with cocaine (1×10^{-4}) relaxed at a rate not significantly different from that of strips exposed to the lower concentration, 33.5% in 5 minutes after oil immersion.

c. _Iproniazid pretreated strips:_ Strips were pretreated with iproniazid as described in Methods and then treated as the control strips. The mean height of contraction of 10 strips before oil immersion was 28.5 mm. Iproniazid pretreatment had no apparent effect on the rate of relaxation of strips contracted with noradrenaline (1×10^{-8}). They relaxed a mean of 50.3% in 5 minutes, compared to 51.0% for control strips.

d. Tropolone pretreated strips: Strips were exposed to tropolone 3×10^{-5} or 1×10^{-4} for 30 to 60 minutes as described in Methods and then treated as the controls. The mean height of contraction of 6 strips before oil immersion was 26.7 mm. Tropolone significantly decreased the relaxation measured 5 minutes after oil immersion, and increased the time for half relaxation to 1.3 times that of the controls.

e. Iproniazid pretreated, cocainized strips: Strips were pretreated with iproniazid and then treated in the same way as the cocainized strips. The mean height of contraction of 8 strips before oil immersion was 33.6 mm. These strips relaxed at a rate not significantly different from that of strips treated with cocaine alone. They relaxed 37.5% in 5 minutes, compared to 39.0 and 50.3% for strips treated only with cocaine or iproniazid, respectively. This slowing was significant at the 2% level of probability.

f. <u>Tropolone pretreated, cocainized strips</u>: Strips were pretreated with tropolone as described in Methods and then treated in the same way as the cocainized strips. The mean height of contraction of 5 strips before oil immersion was 32.4 mm. These strips relaxed at a significantly slower rate than did strips treated with either tropolone or cocaine alone. They relaxed only 20.8% in 5 and 52.8% in 15 minutes. Tropolone pretreated, cocainized strips required a mean of 13.9 minutes to relax 50%, whereas strips treated with cocaine and tropolone alone required only 7.9 and 7.0 minutes, respectively.

g. <u>Iproniazid plus tropolone pretreated strips</u>: Strips were pretreated with iproniazid and then treated in the same way as the tropolone pretreated strips. The mean height of contraction of 7 strips before oil immersion was 30.1 mm. These strips relaxed at a significantly slower rate than did strips pretreated with tropolone alone. They relaxed only 44.9% in 15 minutes, whereas those exposed to tropolone alone relaxed 73.8% in the same interval. The time for 50% relaxation of iproniazid plus tropolone pretreated strips was 17 minutes, compared to 7.0 and 5.1 minutes, respectively, for strips pretreated with tropolone and iproniazid alone.

h. <u>Iproniazid plus tropolone pretreated, cocainized strips</u>: Strips were pretreated with iproniazid and tropolone and then treated in the same way as the cocainized strips. The mean height of contraction of 9 strips before oil immersion was 34.3 mm. These strips relaxed only 10.4% in 5 and 38.7% in 30 minutes. The time to relax 30%, determined by direct measurements, was about 24 minutes, compared to 2.8 minutes for the controls. Cocaine in a concentration of 1×10^{-4} instead of

1×10^{-5} was used in the combined treatment of some strips and these strips relaxed a mean of only 25.0% in 30 minutes.

Strips treated with iproniazid, tropolone and cocaine, but not exposed to any sympathomimetic, shortened very slowly during 30 minutes of oil immersion. However, the magnitude of this change was insufficient to alter the results significantly (Section V, F). None of these agents alone or in pairs altered the baseline of aortic strips, either in Krebs solution or after oil immersion.

2. Choice of Tropolone Concentration:

Preliminary experiments with tropolone showed that the maximal effect on rate of relaxation was exerted by a concentration of 3×10^{-5}. Some experiments included in the results presented in this section were performed with 1×10^{-4}, but 3×10^{-5} was the standard concentration used. A concentration of 3×10^{-4} tropolone occasionally caused a small contraction of the aortic strips, and this stimulant effect limited the use of higher concentrations. A concentration of 1×10^{-4} tropolone occasionally decreased the amplitude of contractions produced by noradrenaline, and higher concentrations caused progressively more severe depression. The exposure to tropolone before testing with an agonist ranged from 30 to 60 minutes, but was usually 30 minutes.

The effects of tropolone in concentrations of 1×10^{-6}, 1 and 3×10^{-5}, and 1×10^{-4} on the rate of relaxation of aortic strips are compared in Table IX. It can be seen that a concentration of 1×10^{-4} exerted no greater effect than did 3×10^{-5} on strips contracted with either noradrenaline (1×10^{-8}) or adrenaline (1×10^{-6}).

TABLE IX

EFFECT OF VARIOUS CONCENTRATIONS OF TROPOLONE ON THE RELAXATION
OF NORADRENALINE AND ADRENALINE CONTRACTED STRIPS IN OIL

Agonist	Conc. of Tropolone	No. of Expts.	PERCENT RELAXATION		
			5 Min.	15 Min.	30 Min.
Noradrenaline (1×10^{-8})	1×10^{-6}	2	47.9 ± 8.2	83.4 ± 4.8	93.5 ± 0.6
	1×10^{-5}	2	30.7 ± 2.7	67.4 ± 0.7	86.9 ± 1.1
	3×10^{-5}	5	21.4 ± 3.9	44.0 ± 6.4	64.6 ± 5.2
	1×10^{-4}	3	21.7 ± 2.3	52.7 ± 4.5	73.8 ± 4.4
Adrenaline (1×10^{-6})	3×10^{-5}	2	1.0 ± 1.0	4.1 ± 2.3	24.4 ± 6.9
	1×10^{-4}	3	1.6 ± 0.8	10.5 ± 1.5	30.7 ± 3.2

All noradrenaline contracted strips were pretreated with
iproniazid. Times refer to period of oil immersion. Incubation
with tropolone before contractions was for 30 to 60 min.

A comparison of various tropolone incubation times also revealed no difference between the effects of 30 and 60 minute periods. Although tropolone has been recently shown to be a competitive rather than a noncompetitive inhibitor of COMT (Mavrides et al., 1963), as was believed earlier, the enzyme appeared to be effectively inhibited by a concentration of 3×10^{-5} even in the presence of a very high concentration of adrenaline.

3. Effects of H 22/54:

Attempts to confirm that the effect of tropolone was due to inhibition of COMT by using other agents reputed to inhibit this enzyme were complicated by various ancillary properties of the available inhibitors. Pyrogallol could not be used effectively because of its instability in alkaline media. H 22/54 is a dopacetamide derivative reported to be a competitive inhibitor of COMT with a potency equivalent to that of 4-methyl tropolone (Corrodi, 1965). Preliminary investigation of this inhibitor showed that it was not suitable for use in these experiments because concentrations as low as 1×10^{-6} markedly reduced the magnitude of subsequent responses to noradrenaline.

4. Effect of Tropolone on the Relaxation of Phenylephrine Contracted

Strips:

To confirm that the observed effect of tropolone on the rate of relaxation of aortic strips contracted by noradrenaline was due to inhibition of COMT, the effect of this agent on the relaxation in oil of phenylephrine contracted strips was studied. Phenylephrine is not a substrate for the transferase. Three strips were contracted with phenylephrine (3×10^{-8}) in the absence of tropolone and their rate of

relaxation in oil measured, and the procedure repeated after pretreatment with tropolone as described in Methods. Before exposure to tropolone mean relaxation was 43.3% in 5 and 71.9% in 15 minutes after oil immersion, and in the presence of the inhibitor 39.4% in 5 and 71.3% in 15 minutes. The rates did not differ even at the 10% level of probability when compared by the $\underline{t}$ test for paired data.

5.   Effect of Pretreatment with Cocaine on Contraction Amplitude:

In most of the experiments described in this thesis cocaine was added to the muscle chamber after the response to an agonist had reached a plateau. A comparable potentiation of responses to noradrenaline was observed when cocaine was added to the chambers before the agonist. Figure 22 shows mean log dose-response curves for noradrenaline determined on 3 control strips and 3 treated with cocaine (1×10^{-5}). These were selected as having similar sensitivities to noradrenaline on the basis of preliminary threshold responses. Responses to noradrenaline (1×10^{-8}) were increased a mean of 7.4 mm, 22.9% of the control contraction height. This is slightly less than equivalent to tripling the concentration of noradrenaline. At any point on the curve the response in the presence of cocaine (1×10^{-5}) appears to be equivalent to that produced by a 2 to 3 times higher concentration of noradrenaline alone.

6.   Effects of Various Cocaine Exposure Times on the Relaxation of

Noradrenaline Contracted Strips:

The effects of various periods of incubation with cocaine were studied to determine whether the standard 10 to 20 minute exposure before oil immersion produced the maximal effect of this agent
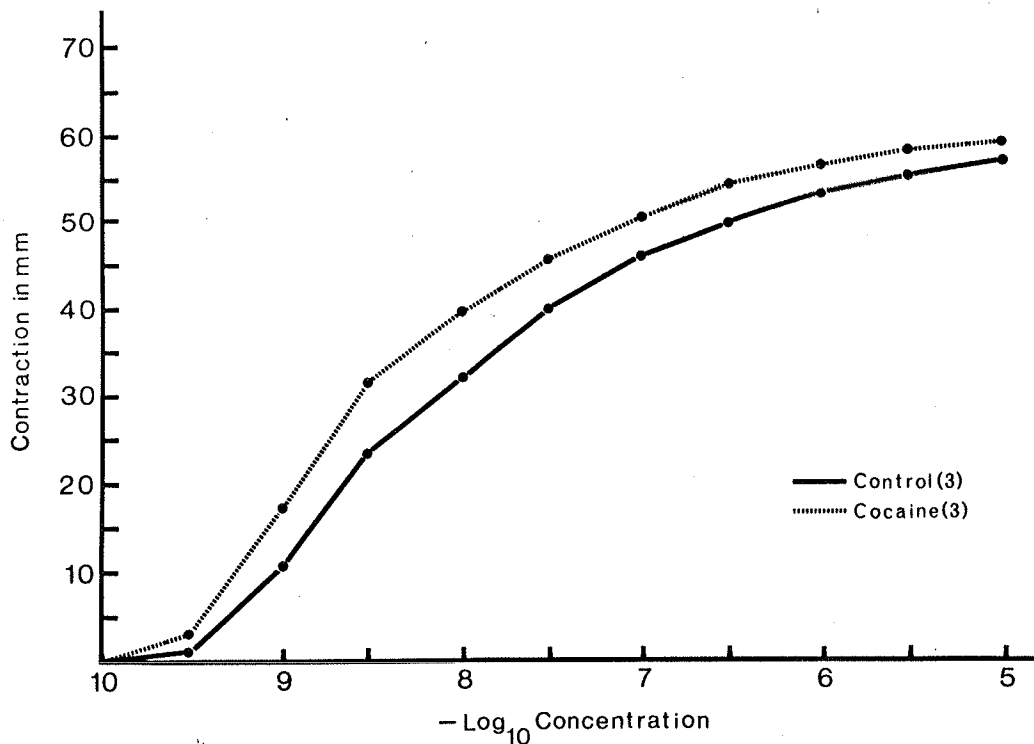
Fig. 22.   Effect of Pretreatment with Cocaine on the Noradrenaline
Log Dose-Response Curve.

Figures in parentheses indicate the number of complete
experiments on which each curve is based. Cocaine concentration
was 1×10^{-5}.

on rates of relaxation. Each of 4 aortic strips from reserpinized
animals was exposed twice to cocaine (1 X 10^{-4}), for 10 and 30
minutes, in random order, contracted with noradrenaline (1 X 10^{-8}),
and after each contraction had reached a plateau (about 10 minutes),
immersed in oil. The results are shown in figure 23. It can be seen
that cocaine had essentially the same effect whether present for totals
of 20 or of 40 minutes before oil immersion. There is also no apparent
difference between these results and those of experiments in which
cocaine (1 X 10^{-4}) was added to the chambers 10 to 20 minutes before
oil immersion, after contractions produced by noradrenaline had reached
a plateau. Strips incubated with cocaine for 30 minutes before the
addition of noradrenaline relaxed a mean of 27.2% and those treated
with cocaine after noradrenaline a mean of 28.8% in 5 minutes.

7. Effects of Tropolone and Iproniazid on Contraction Amplitude:

The effects of the enzyme inhibitors on contractile responses
were determined by comparing the maximal contraction amplitude in
response to noradrenaline (1 X 10^{-8}) of all tropolone pretreated
strips, including those which subsequently received cocaine, with the
peak responses of control and iproniazid pretreated strips (Table X).
The peak contraction of 11 tropolone pretreated strips was 28.4 $\pm$
2.4 mm, and that of 20 control strips and 18 pretreated with iproniazid
27.5 $\pm$ 2.0 mm and 29.1 $\pm$ 2.4 mm, respectively. The peak response of
strips pretreated with both iproniazid and tropolone was 30.4 $\pm$ 2.0 mm.
There were no significant differences between any of these groups. A
separate study which compared responses of the same strips to noradren-
aline before and after exposure to iproniazid, also showed no effect on
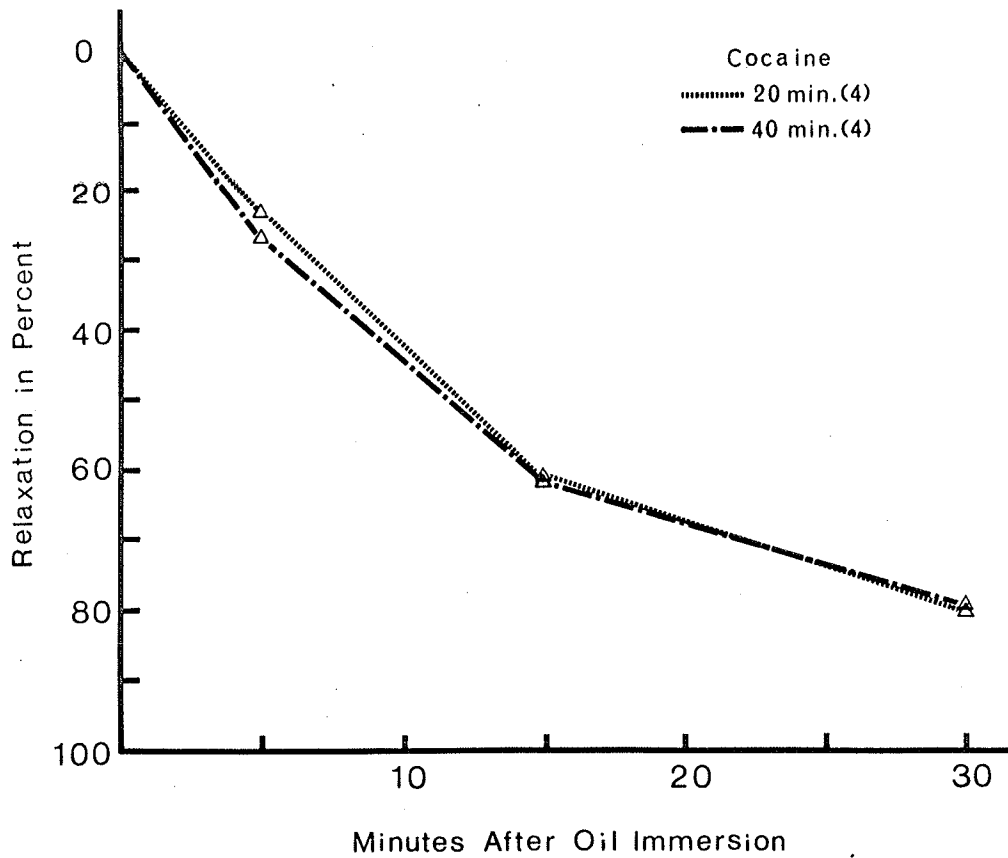
Fig. 23. Effect of Duration of Exposure to Cocaine on the Relaxation of Noradrenaline Contracted Aortic Strips after Oil Immersion.

Time is the total exposure to cocaine (1×10^{-4}) before oil immersion. Noradrenaline concentration was 1×10^{-8}. Figures in parentheses indicate number of preparations represented by each curve.

TABLE X

EFFECTS OF IPRONIAZID, TROPOLONE AND COCAINE ON

NORADRENALINE (1×10^{-8}) CONTRACTION AMPLITUDE

Treatment	Noradrenaline Contraction (mm)	Cocaine Increment				Equiv. Conc. Noradrenaline	
		1×10^{-5}*		1×10^{-4}*		1×10^{-5}*	1×10^{-4}*
		mm	%	mm	%		
Control	27.5 ± 2.0 (20)	4.6 (10)	18.3 ± 1.6	6.8 (4)	25.3 ± 5.2	2.2×10^{-8}	3.0×10^{-8}
Iproniazid	29.1 ± 2.4 (18)	5.0 (8)	19.3 ± 3.7	--	---	---	---
Tropolone	28.4 ± 2.4 (11)	4.4 (5)	16.0 ± 2.0	--	---	---	---
Iproniazid + Tropolone	30.4 ± 2.0 (16)	4.4 (9)	15.6 ± 2.3	4.0 (6)	13.7 ± 2.0	---	1.8×10^{-8}

* Concentration of Cocaine

All heights refer to peak heights. Equivalent concentrations of noradrenaline are shown for control cocainized strips and for all groups which differ significantly. Contraction amplitudes of iproniazid, tropolone and iproniazid plus tropolone treated strips were not significantly different from the control.

Statistical analysis (cocaine increments):

Cocaine (1×10^{-5}) vs. Cocaine (1×10^{-4}) $\quad\quad$ $P < 0.2 > 0.1$

Cocaine (1×10^{-4}) vs. Iproniazid + tropolone + cocaine (1×10^{-4}) $\quad\quad$ $P < 0.05$

amplitude of contraction (Section III, A, 1).

8.   Effect of Cocaine on Contraction Amplitude:

Cocaine (1×10^{-5}) was added to the chamber after each

response to noradrenaline (1×10^{-8}) had reached a plateau height

and, as in the studies with phenylephrine, the increment produced

calculated as a percent of the immediately preceding amplitude.

Table X shows the potentiation produced by cocaine, alone and in the

presence of the enzyme inhibitors iproniazid and tropolone. The

contraction of control strips was increased 4.6 mm ($18.3 \pm 1.6\%$).

Iproniazid did not alter the magnitude of the cocaine effect. Pre-

treatment with tropolone, alone or in combination with iproniazid,

tended to decrease the percent increment produced by cocaine, but the

differences from the response of control strips were not statistically

significant. Cocaine augmented the contraction of strips pretreated

with both iproniazid and tropolone by 4.4 mm ($15.6 \pm 2.3\%$). The

potentiation produced by cocaine (1×10^{-4}) is also shown in Table X.

As a basis for determining the concentrations of noradrena-

line alone required to produce contractions equal to those due to

noradrenaline plus cocaine, a noradrenaline dose-response curve was

constructed from the responses of a series of aortic strips (Fig. 1).

It was determined from this graph that the effect of cocaine

(1×10^{-5}) was approximately equivalent to doubling the noradrenaline

concentration.

C. ADRENALINE (1 X 10^{-8}) CONTRACTED AORTIC STRIPS

1. Relaxation in Oil:

A concentration of 1 X 10^{-8} adrenaline was selected for

study with the oil immersion technique because it produced contractions

comparable in magnitude to those produced by noradrenaline (1 X 10^{-8})

and phenylephrine (3 X 10^{-8}). The mean contraction height of 6 control

strips before oil immersion was 24.7 mm, and only 6 of 48 strips,

subjected to various pretreatments, responded to this concentration of

adrenaline with contractions of less than 20 mm.

The same 8 treatment conditions employed in the study of

noradrenaline were used to investigate mechanisms for the inactivation

of adrenaline. Mean relaxation curves for all groups are shown in

figure 24 (a and b), and individual records typical of several of the

treatment conditions are shown in figure 21. The time required for

each strip to relax 50% was directly measured and the mean time

calculated for each treatment condition. These results are summarized

in Table VIII.

a. Control strips: Strips contracted by adrenaline

(1 X 10^{-8}) relaxed in oil at a rate comparable to that of strips

contracted by the same concentration of noradrenaline, the process

being 55.1% and 51.0% complete, respectively, in 5 minutes. Strips

contracted with adrenaline relaxed a mean of 80.8% in 15 minutes.

b. Cocainized strips: The mean height of contraction of

5 strips prior to oil immersion was 29.4 mm. Cocaine (1 X 10^{-5})

clearly slowed the rate of relaxation of some strips when each was

compared to a control strip cut from the same aorta. Because of the

Fig. 24, a and b.   Effects of Iproniazid, Tropolone and Cocaine on Relaxation of Adrenaline Contracted Aortic Strips in Oil.

Treatment with iproniazid (IPN) and tropolone (Trop) was as described in Methods.   Concentration of adrenaline was 1×10^{-8} and that of cocaine 1×10^{-5} in all experiments.   Figures in parentheses indicate the number of preparations represented by each curve.   Bars represent the standard errors of means.   See text for detailed description of experiments.

Statistical Analysis:

	Min.
Control vs. Cocaine	$5 - P < 0.2$
Control vs. Iproniazid	N.S.
Control vs. Tropolone	$5 - P < 0.001$ $15 - P < 0.001$ $30 - P < 0.05$
Iproniazid + Cocaine vs. Cocaine	N.S.
Tropolone + Cocaine vs. Tropolone	$5 - P < 0.05$ $15 - P < 0.01$ $30 - P < 0.01$
Iproniazid + Tropolone vs. Tropolone	$5 - P < 0.02$ $15 - P < 0.001$ $30 - P < 0.01$
Iproniazid + Tropolone vs. Iproniazid + Tropolone + Cocaine	$5 - P < 0.1$ $15 - P < 0.01$ $30 - P < 0.01$

a.

Control(6) ----IPN(6)
Cocaine(5) ----Trop(7)
wwwwwIPN+Trop+Cocaine(6)

Releaxation in Percent

Minutes After Oil Immersion

b.

----IPN(6)
..........Cocaine(5)
.....IPN+Cocaine(5)
.....Trop+Cocaine(6)
.....IPN+Trop(7)

Relaxation in Percent

Minutes After Oil Immersion
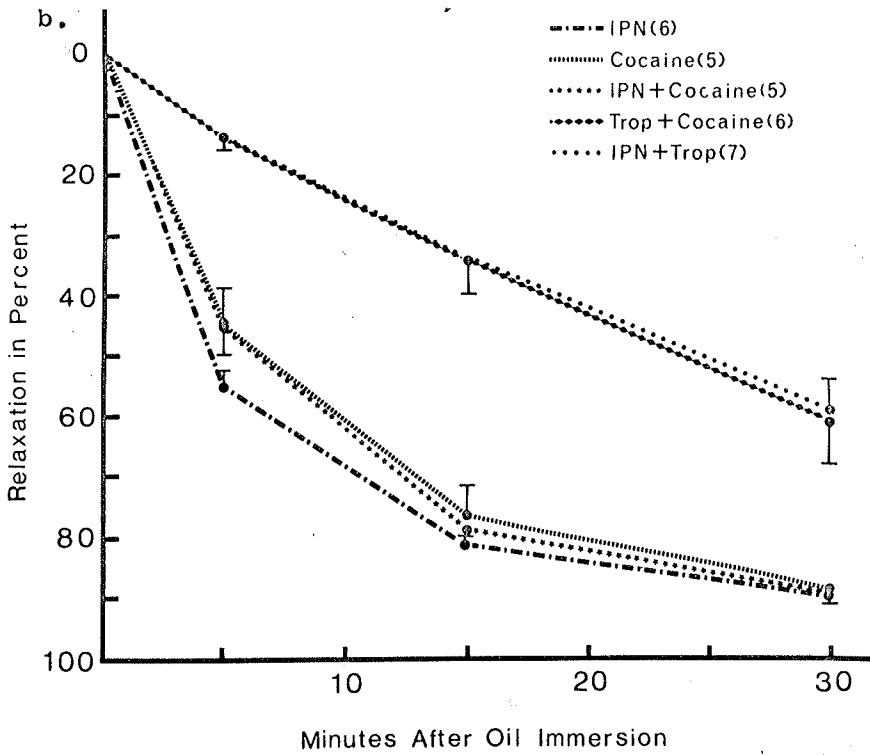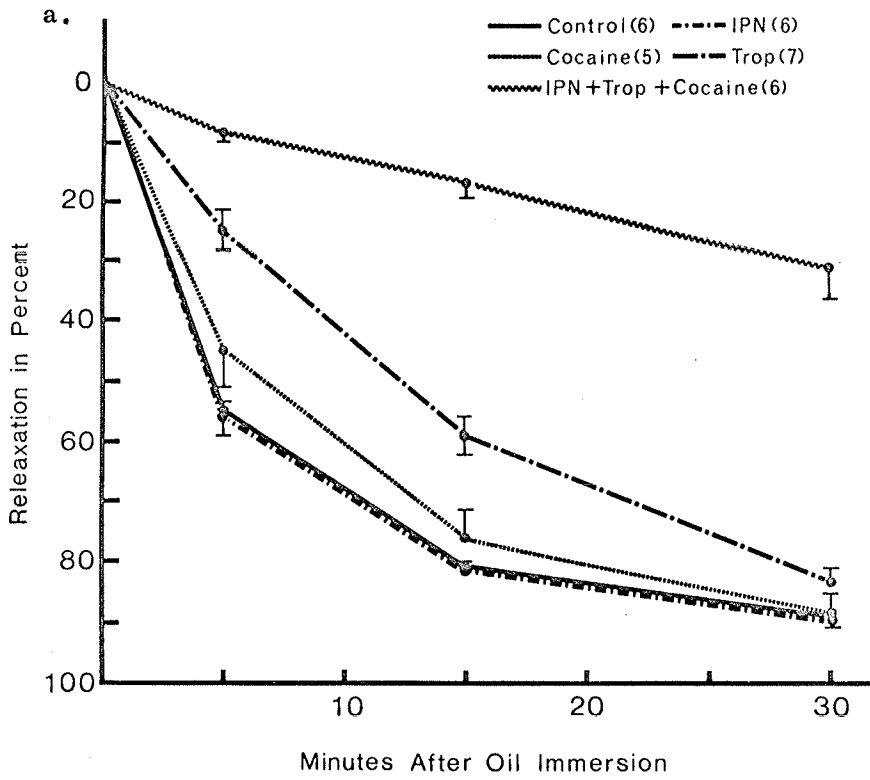
small number of strips used and the comparatively large variance, the significance of this effect could not be established by the two-tailed t test for unpaired data which was employed routinely. However, by the one-tailed test, the slowing due to cocaine, measured 5 minutes after oil immersion, was significant at the 10% level of probability. Cocaine (1×10^{-5}) increased the time required for 50% relaxation to 6.6 minutes, about 1.6 times the control value of 4.2 minutes. The time required for noradrenaline (1×10^{-8}) contracted strips to relax 50% was increased to about 1.5 times that of the controls by the same concentration of cocaine.

c. Iproniazid pretreated strips: The mean height of contraction of 6 strips before oil immersion was 24.0 mm. Pretreatment with iproniazid did not alter the rate of relaxation of strips contracted by adrenaline (1×10^{-8}). Relaxation was 55.6% complete in 5 minutes, compared to 55.1% for the controls, and was 81.2% complete 15 minutes after oil immersion.

d. Tropolone pretreated strips: The mean height of contraction of 7 strips before oil immersion was 28.1 mm. Tropolone slowed the relaxation of strips contracted by adrenaline more than that of those contracted by noradrenaline, the process being 24.7% and 41.0%. complete, respectively, 5 minutes after oil immersion. Tropolone pretreatment increased the time required for 50% relaxation of strips contracted by adrenaline (1×10^{-8}) and noradrenaline (1×10^{-8}) to about 2.9 and about 1.3 times that of their controls, respectively.

e. Iproniazid pretreated, cocainized strips. The mean height of contraction of 5 strips before oil immersion was 29.6 mm.

These strips relaxed at a rate not significantly different from that
of strips treated with cocaine alone. Relaxation was 45.2% complete
in 5 and 78.6% complete in 15 minutes. The combination of iproniazid
and cocaine (1×10^{-5}) increased the time required for half relaxation
of strips contracted by noradrenaline (1×10^{-8}) and adrenaline
(1×10^{-8}) to about 1.5 and 1.3 times that of their controls, respec-
tively.

      f. Tropolone pretreated, cocainized strips: The mean
height of contraction of 6 strips before oil immersion was 36.7 mm.
These strips relaxed at a distinctly slower rate than did those treated
with tropolone or cocaine alone, the process being 13.9%, 24.7% and
44.8% complete, respectively, 5 minutes after oil immersion. The
combination of tropolone and cocaine slowed the relaxation of adren-
aline contracted more than that of noradrenaline contracted strips;
the time for 50% relaxation being 5.9 and 2.6 times that of their
controls, respectively.

      g. Iproniazid plus tropolone pretreated strips: The mean
height of contraction of 7 strips before oil immersion was 29.4 mm.
These strips relaxed at a significantly slower rate than did those
pretreated with tropolone alone, 13.6% in 5, 33.9% in 15 and 59.0% in
30 minutes after oil immersion. Strips pretreated with tropolone alone
relaxed 83.6% in 30 minutes. The combination of iproniazid and
tropolone slowed the relaxation of adrenaline contracted more than it
did that of noradrenaline contracted strips, the time required for
50% relaxation being 6.1 and 3.2 times that of their controls,
respectively.

h. Iproniazid plus tropolone pretreated, cocainized strips.

The mean height of contraction of 6 strips before oil immersion was

34.5 mm. These strips relaxed at a significantly slower rate than did

those pretreated with iproniazid plus tropolone, only 16.9% in 15

minutes. The marked decrease in the rate of relaxation of strips

contracted by adrenaline (1×10^{-8}) produced by the combination of

iproniazid, tropolone and cocaine was similar to that produced by these

agents in strips contracted by noradrenaline (1×10^{-8}).

2. Effects of Tropolone and Iproniazid on the Magnitude of Responses
   to Adrenaline:

The effects of tropolone and iproniazid on the magnitude of

the contractile response to adrenaline (1×10^{-8}) were determined as

described in the study of noradrenaline. (See Table XI.) The peak

response of 13 control strips was 24.4 $\pm$ 1.9 mm and that of 15 strips

pretreated with tropolone 32.9 $\pm$ 2.1 mm, a difference significant at

the 1% level of probability. Fourteen strips pretreated with both

iproniazid and tropolone responded with a mean peak contraction of

31.6 $\pm$ 2.4 mm, which did not differ significantly from that of strips

pretreated with tropolone alone. Pretreatment with iproniazid alone

did not alter the magnitude of responses to adrenaline (1×10^{-8}); the

peak contraction of 12 strips being 24.3 $\pm$ 1.8 mm.

3. Effects of Tropolone and Cocaine on Responses to Isoproterenol:

Pretreatment of aortic strips with tropolone increased the

magnitude of responses to adrenaline (1×10^{-8}) but not that of

responses to noradrenaline (1×10^{-8}). Responses to a third substrate

of COMT, isoproterenol, were studied in an attempt to confirm that

TABLE XI

EFFECTS OF IPRONIAZID, TROPOLONE AND COCAINE

ON ADRENALINE CONTRACTION AMPLITUDE

Treatment	Adrenaline (1×10^{-8}) (mm)	Equiv. Conc. Adrenaline	Cocaine Increment 1×10^{-5}		Equiv. Conc. Adrenaline
			mm	%	
Control	24.4 ± 1.9 (13)	1×10^{-8}	5.0 (5)	21.1 ± 3.2	2.1×10^{-8}
Iproniazid	24.3 ± 1.8 (12)	--	5.1 (5)	21.9 ± 3.2	--
Tropolone	32.9 ± 2.1 (15)	3.2×10^{-8}	4.3 (6)	13.9 ± 2.6	7.0×10^{-8}
Iproniazid + Tropolone	31.6 ± 2.4 (14)	2.6×10^{-8}	3.8 (6)	13.3 ± 2.6	5.1×10^{-8}

All heights refer to peak heights. Equivalent concentrations of adrenaline are shown for control strips and for all groups which differ significantly.

Statistical Analysis:

Response amplitude

Control vs. tropolone $\quad P < 0.01$
Control vs. Iproniazid $\quad P < 0.05$
+ tropolone

Cocaine increment

Cocaine vs. tropolone $\quad P < 0.2 > 0.1$
Cocaine vs. iproniazid $\quad P < 0.1 > 0.05$
+ tropolone

enzyme inhibition was responsible for the observed potentiation. Isoproterenol, in low to moderate concentrations (1×10^{-9} to 1×10^{-7}), relaxes aortic strips contracted by a variety of agents through an action on β adrenergic receptors. Thus, potentiation of responses to isoproterenol is recorded as an increase in the relaxation produced by a given concentration. The results of these experiments are shown in figure 25.

Strips were contracted by noradrenaline (1×10^{-8}) and the relaxation produced by progressively increasing concentrations of isoproterenol recorded (Fig. 25, a). Control responses to the cumulative addition of isoproterenol (3×10^{-9} to 1×10^{-7}) are shown in the record on the left. After washout and recovery of the strip, tropolone (3×10^{-5}) was added to the chamber and the test with noradrenaline and isoproterenol repeated 30 minutes later (a, center). It can be clearly seen that tropolone enhanced the responses to isoproterenol and decreased the concentration required for maximal relaxation by this agent. After the tropolone had been washed out, a second control test showed that the response to isoproterenol had returned toward the initial level (a, right). (Note particularly the difference in the concentration required to produce maximal inhibition.) The presence of some residual tropolone might account for the somewhat greater responses to isoproterenol in the second than in the first control test.

Figure 25 (b) shows the results obtained in a similar experiment in which phenylephrine rather than noradrenaline was used to contract the aortic strip. This sympathomimetic amine is not a sub-

a.

NA   NA   NA

b.

P   P   P

c.

P   P   P
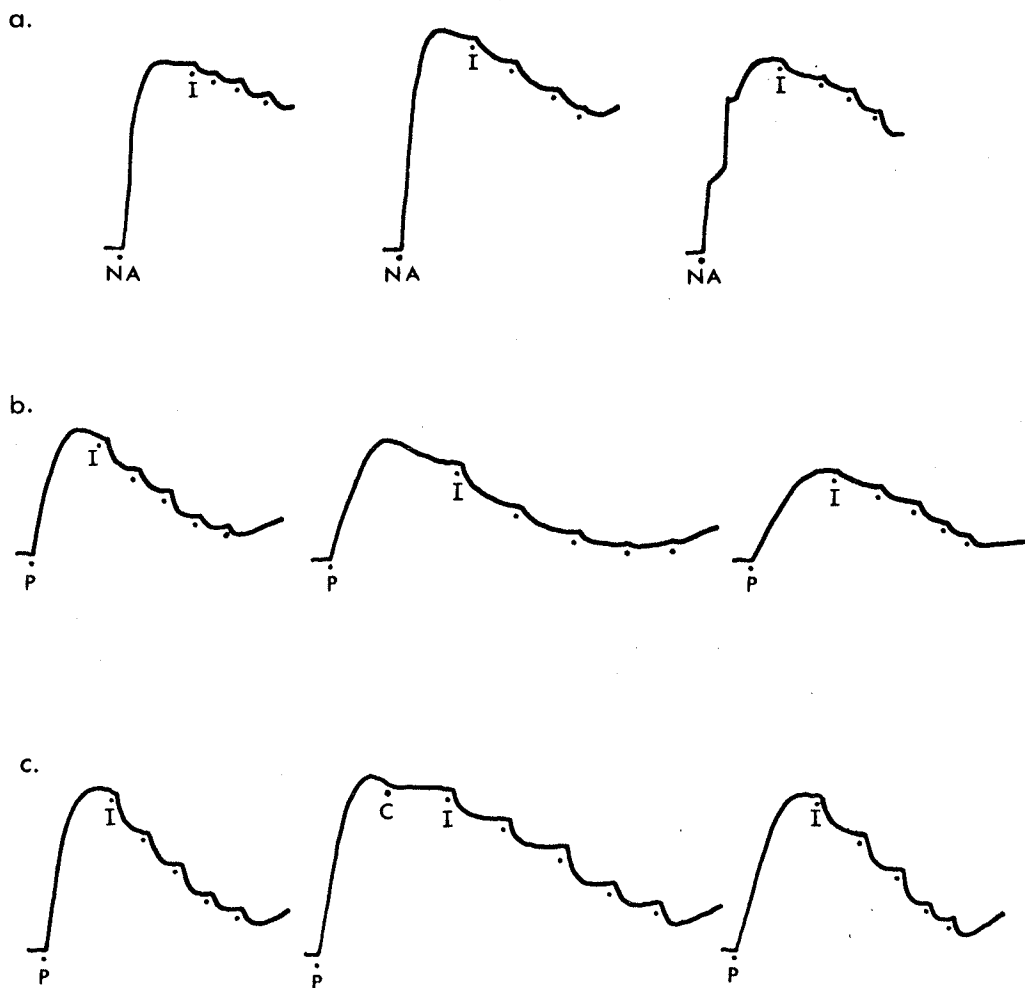
Fig. 25. Effects of Tropolone and Cocaine on Isoproterenol-Induced Relaxation of Aortic Strips.

a. Responses of a noradrenaline (NA) (1×10^{-8}) contracted strip to cumulative concentrations of isoproterenol (I) (3×10^{-9}, 1 and 3×10^{-8}, 1×10^{-7}) (dots) control responses; center, responses in the presence of tropolone (3×10^{-5}); right, responses after washout of tropolone. (The irregularity in the record during contraction is a drum artifact).
b. Responses to the same procedures as in a, except that the strip was contracted by phenylephrine (P) (3×10^{-8}) and the cumulative concentrations of isoproterenol were 1 and 3×10^{-9}, 1 and 3×10^{-8}, 1×10^{-7}.
c. Responses of a strip contracted by phenylephrine and relaxed by isoproterenol as in b. Left, control responses; center, responses to isoproterenol in the presence of cocaine (C) (1×10^{-5}); right, responses after washout of cocaine.

strate for COMT. It can be seen that tropolone (3×10^{-5}) potentiated

the action of isoproterenol (1×10^{-9} to 1×10^{-7}) (center record),

and that this effect was markedly decreased after the tropolone was

washed out. Figure 25 (c) shows the responses of a strip from the

same aorta also contracted by phenylephrine and relaxed by isoproterenol,

but treated with cocaine (center record) rather than tropolone. Cocaine

(1×10^{-5}) did not increase the responses to isoproterenol and did not

decrease the concentration required for maximal relaxation. The

observation that cocaine does not potentiate the inhibitory effect of

isoproterenol on aortic strips is in agreement with the findings of

Furchgott (1955).

4. Effect of Cocaine on Contraction Amplitude:

Observations on the potentiation of responses to adrenaline

by cocaine alone and after pretreatment with iproniazid and tropolone

are summarized in Table XI. The responses of control strips were

increased 5.0 mm ($21.2 \pm 3.2\%$) by cocaine (1×10^{-5}). Iproniazid did

not alter the magnitude of the potentiation by cocaine, as was found in

the studies with noradrenaline. Pretreatment with tropolone, alone or

in combination with iproniazid, tended to decrease the effect of cocaine,

in terms of absolute increment, but the difference was not significant

at the 5% level of probability. However, as shown in Table XI, when

the values were corrected for the potentiation already produced by the

tropolone, it was found that potentiation by cocaine was unaffected by

pretreatment with enzyme inhibitors. In all groups the effect of co-

caine was approximately equivalent to that of doubling the concentra-

tion of adrenaline.

D. NORADRENALINE (1×10^{-6}) CONTRACTED AORTIC STRIPS

1. Relaxation in Oil:

The COMT inhibitor tropolone significantly slowed the

relaxation of aortic strips contracted by noradrenaline (1×10^{-8}),

whereas the MAO inhibitor iproniazid did not. The effects of these

enzyme inhibitors on rates of relaxation were also studied in aortic

strips contracted by a 100 times higher concentration of noradrenaline

to determine the extent to which the observed changes are dependent on

agonist (substrate) concentration. Twelve strips cut from 3 aortas

were used for these experiments. One strip from each aorta was

subjected to each of the 4 treatments described below. Mean relaxation

curves from all experiments are shown in figure 26, and records from a

typical experiment in figure 27. All strips were exposed to noradren-

aline (1×10^{-6}) for 30 minutes and the chambers then drained and

filled with oil. The procedures for enzyme inhibition were as described

in Methods.

a. Control strips: The mean height of contraction of 3 strips

prior to oil immersion was 55.7 mm. These strips relaxed 14.3% in 5,

52.4% in 15 and 77.1% in 30 minutes after oil immersion.

b. Iproniazid pretreated strips: The mean height of con-

traction of 3 strips prior to oil immersion was 58.0 mm. In contrast

to the results obtained in the study with the lower concentration of

noradrenaline, strips pretreated with iproniazid and contracted by

noradrenaline (1×10^{-6}) relaxed significantly slower than did their

controls. Relaxation was only 2.2% complete in 5, 16.0% in 15 and

42.5% in 30 minutes after oil immersion. The time required for these
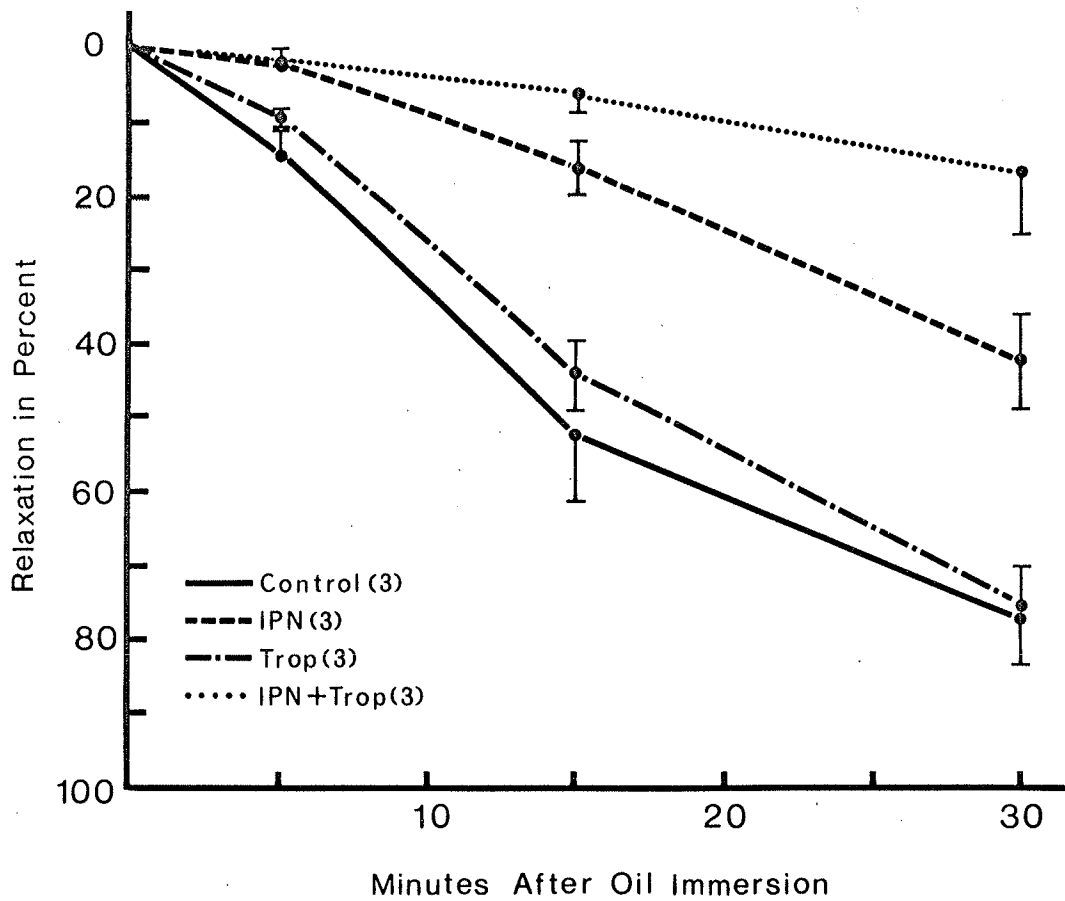
Fig. 26. Effects of Iproniazid and Tropolone on Relaxation in Oil of Aortic Strips Contracted by a High Concentration of Noradrenaline.

Pretreatment with iproniazid (IPN) or tropolone (Trop) before contraction by noradrenaline (1×10^{-6}). Figures in parentheses indicate number of preparations represented by each curve. Statistical analysis:

	Min.
Iproniazid vs. Control	$5 - P < 0.05$
	$15 - P < 0.05$
	$30 - P < 0.02$
Tropolone vs. Control	N.S.
Iproniazid + Tropolone vs. Iproniazid	$15 - P < 0.1$
	$30 - P < 0.1$
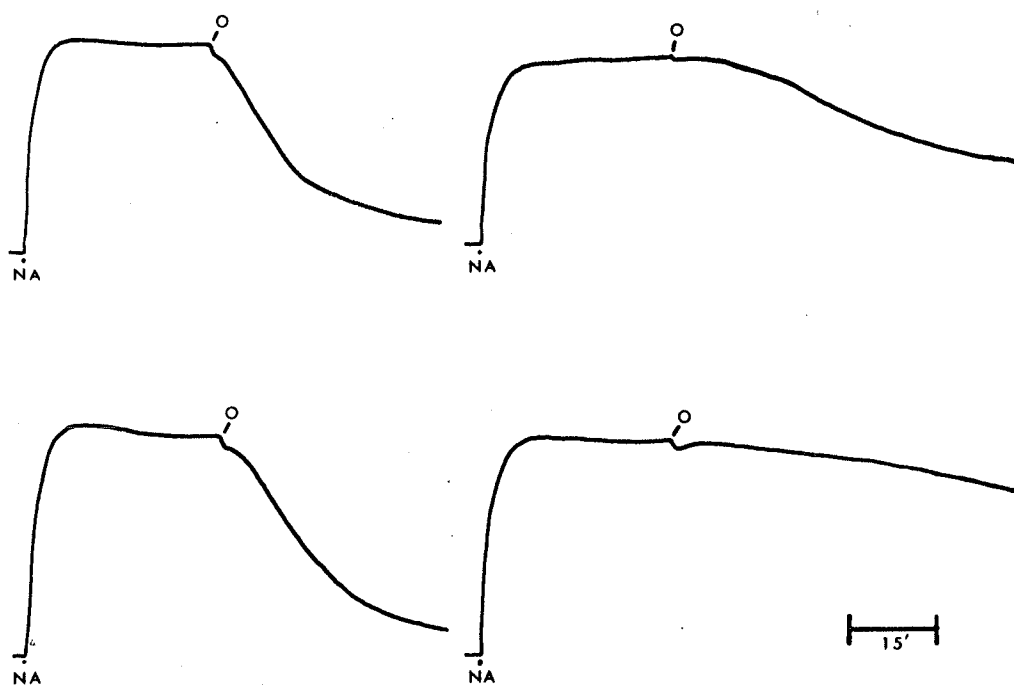
Fig. 27. Effects of Iproniazid and Tropolone on Relaxation
in Oil of Aortic Strips Contracted by a High Concentration of
Noradrenaline.

Responses of 4 strips from the same aorta contracted with
noradrenaline (NA) (1×10^{-6}), followed by oil immersion (O).
Upper records: left, control strip; right, iproniazid pretreated
strip. Lower records: left, tropolone pretreated strip; right,
iproniazid plus tropolone pretreated strip.

strips to relax 50% was increased to about 2.7 times that of their controls.

c. Tropolone pretreated strips: The mean height of contraction of 3 strips prior to oil immersion was 53.7 mm. Relaxation was 9.4% complete in 5, 44.3% in 15 and 75.3% in 30 minutes after oil immersion. With the small number of experiments performed, the difference between this rate and that of the controls could not be shown to be statistically significant. Significance might have been demonstrated with a larger number of preparations. However, it is clear that the effect of COMT inhibition was relatively small (Fig. 26), the time required for half relaxation being only 1.2 times that of the controls. These results demonstrated that the relative importance of the 2 enzymatic pathways for catecholamine inactivation was reversed when the concentration of noradrenaline was increased 100 times.

d. Iproniazid plus tropolone pretreated strips: The mean height of contraction of 3 strips prior to oil immersion was 49.7 mm. Strips pretreated with both iproniazid and tropolone relaxed more slowly than did strips pretreated with iproniazid alone, only 1.9% in 5, 5.8% in 15 and 16.8% in 30 minutes after oil immersion. However, with the small number of experiments, this effect differed from that of the single inhibitor only at the 10% level of probability. The time required for 20% relaxation (50% was not reached during the period of observation) was about 1.9 times that of the strips pretreated with iproniazid alone. The times for 20% relaxation were increased by iproniazid, tropolone and iproniazid plus tropolone to about 2.7, 1.2 and 5.0 times that of the controls, respectively.

E.   ADRENALINE (1 X 10^{-6}) CONTRACTED AORTIC STRIPS

1.   Relaxation in Oil:

In studies employing a low concentration of agonists

(1 X 10^{-8}), tropolone slowed the relaxation of aortic strips con-

tracted by adrenaline more than that of strips contracted by noradren-

aline.   Iproniazid did not affect the relaxation of strips contracted

by either catecholamine.   The relaxation in oil of strips contracted by

adrenaline (1 X 10^{-6}) was studied in the same way as that of strips

contracted by noradrenaline (1 X 10^{-6}) to determine if the relative

importance of enzymatic inactivation pathways for adrenaline changed

with agonist concentration as was found in the case of noradrenaline.

Eighteen strips from 5 animals were exposed to adrenaline (1 X 10^{-6})

for 30 minutes, alone or following enzyme inhibition, and the

chambers then drained and filled with oil.   Mean relaxation curves for

the 4 treatment groups are shown in figure 28.

a.   Control strips: The mean height of contraction of 4

strips before oil immersion was 56.0 mm.   These strips relaxed 6.1% in

5, 45.8% in 15 and 79.9% in 30 minutes after oil immersion.

b.   Iproniazid pretreated strips: The mean height of con-

traction of 4 strips before oil immersion was 48.8 mm.   These strips

relaxed 2.6% in 5, 23.3% in 15 and 54.9% in 30 minutes.   The rate of

relaxation of iproniazid pretreated strips, measured 15 and 30 minutes

after oil immersion, was slower than that of the controls at the 10%

level of probability.   The mean difference was considerable, but the

statistical significance was limited by the large variance and the

small number of strips.   Pretreatment with iproniazid increased the
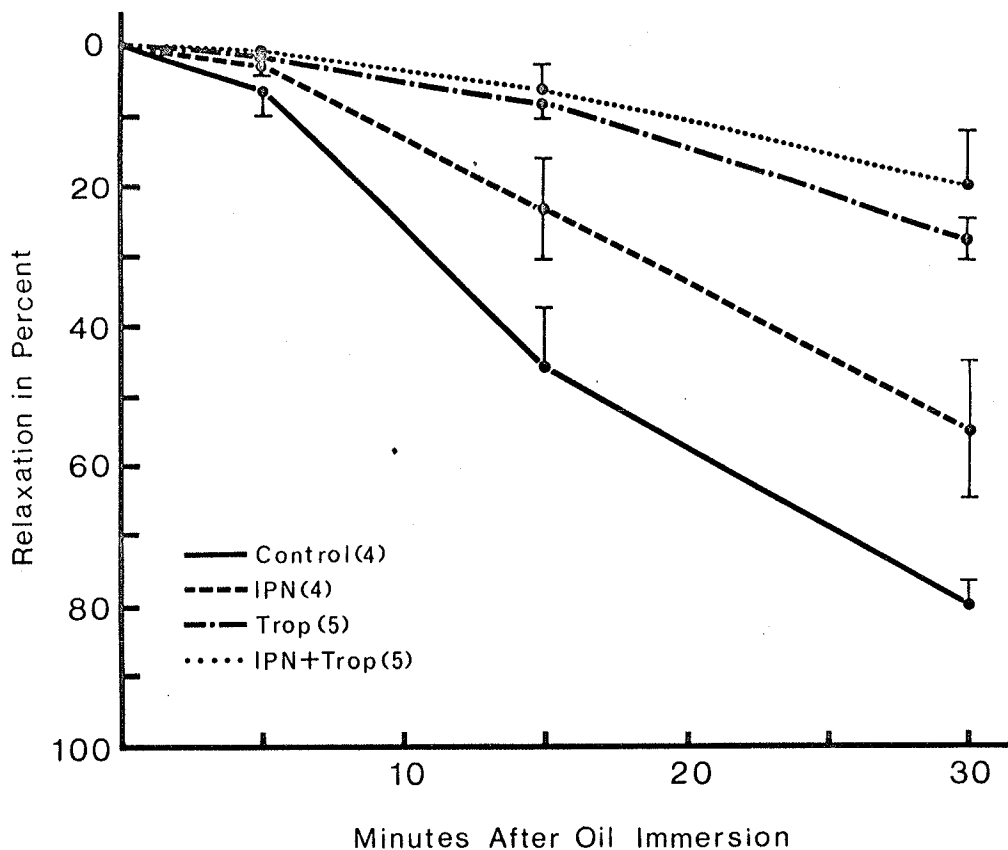
Fig. 28. Effects of Iproniazid and Tropolone on Relaxation in Oil of Aortic Strips Contracted by a High Concentration of Adrenaline.

Pretreatment with iproniazid (IPN) or tropolone (Trop) before contraction with adrenaline (1×10^{-6}). Figures in parentheses indicate number of preparations represented by each curve. Bars indicate standard errors of means.
Statistical analysis:

	Min.
Iproniazid vs. Control	15 - P < 0.1
	30 - P < 0.1
Tropolone vs. Control	5 - P < 0.2
	15 - P < 0.01
	30 - P < 0.001
Iproniazid + Tropolone vs. Tropolone	N.S.

time required for half relaxation of strips contracted by adrenaline
(1×10^{-6}) to less than 1.7, and that of strips contracted by the
same concentration of noradrenaline to about 2.7 times that of their
respective controls.

      c. <u>Tropolone pretreated strips</u>: The mean height of con-
traction of 5 strips before oil immersion was 49.4 mm. These strips
relaxed 7.9% in 15 and 28.1% in 30 minutes after oil immersion. These
values are very significantly ($P < 0.01$ and < 0.001, respectively)
lower than those of their controls. This is in marked contrast to the
slight effect of tropolone on the rate of relaxation of strips con-
tracted by the same high concentration of noradrenaline, the time re-
quired for 50% relaxation being increased to 2.7 vs. only about 1.2
times that of the adrenaline and noradrenaline controls, respectively.
The relative increase in the time for half relaxation produced by
tropolone was about equal to its effect on the relaxation of strips
contracted by a concentration of adrenaline only 1% as great.

      d. <u>Iproniazid plus tropolone pretreated strips</u>: The mean
height of contraction of 5 strips before oil immersion was 53.2 mm.
Relaxation was 6.5 and 19.8% complete in 15 and 30 minutes after oil
immersion, values not significantly different from the 7.9% and 28.1%
relaxation, at the same time intervals, of strips pretreated with
tropolone alone. However, because of the comparatively large variance
and small number of strips, a small effect of the added iproniazid on
relaxation rate cannot be ruled out. The time for 20% relaxation was
increased by iproniazid, tropolone and iproniazid plus tropolone to
about 1.6, 2.9 and 3.5 times that of the controls, respectively.

F. DRUGS BELIEVED TO ACT THROUGH THE RELEASE
OF ENDOGENOUS NORADRENALINE

1. Behavior in Oil of Aortic Strips Exposed to Tyramine and Bretylium:

Tyramine is a sympathomimetic amine which is believed by most
workers to produce its major effects on smooth muscle through the
release of noradrenaline from endogenous nervous structures. (See
Historical Introduction.) It is an excellent substrate for MAO (Hare,
1928; Blaschko, 1937b), and responses of aortic strips to this agent
were found to be markedly potentiated by pretreatment with iproniazid
(Section V, A, 6).

Aortic strips contracted by tyramine gradually relaxed after
oil immersion. A typical response and, for comparison, the relaxation
in oil of an aortic strip contracted by noradrenaline are shown in
figure 29a. However, the contraction amplitude of strips exposed to
tyramine after pretreatment with iproniazid consistently increased
after oil immersion (Fig. 29b and Fig. 10c), and then maintained the
increased contraction for at least 60 minutes. This is in contrast
to the lack of effect of iproniazid pretreatment on the rate of
relaxation of noradrenaline contracted strips in oil (Fig. 29b).

As a working hypothesis, it was assumed that the progressive
increase in the response of tyramine contracted aortic strips in oil
is due to released noradrenaline which is retained in the tissue by
the oil barrier. The effects of bretylium, a compound reported also
to release endogenous catecholamines (Gaffney, 1961; Kirpekar and
Furchgott, 1964), were studied to test this hypothesis.

Bretylium (1 X 10^{-4}) produced only small contractions of

a.

b.
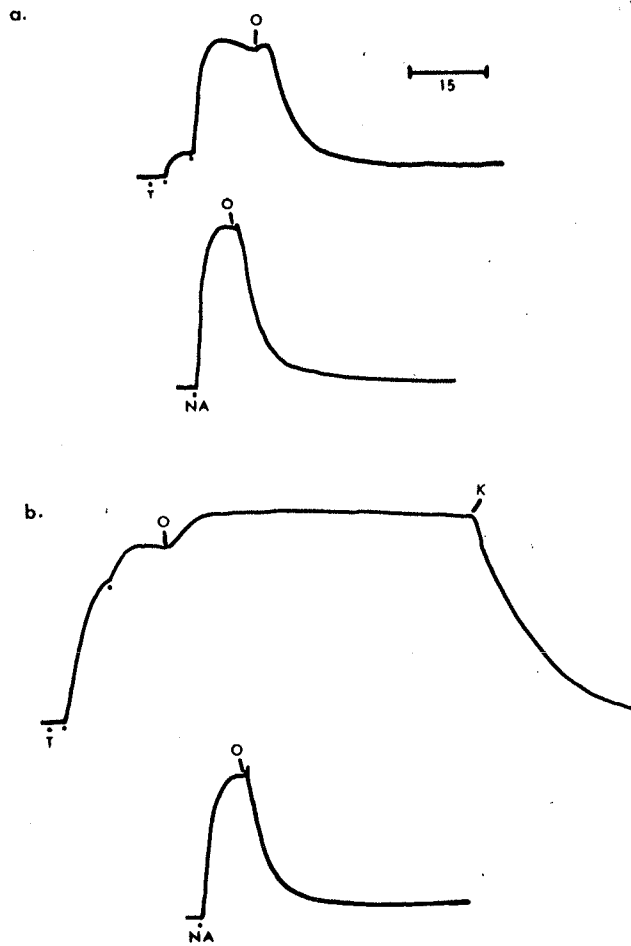
Fig. 29. Effects of Iproniazid on Responses of Aortic Strips to Tyramine.

a. Upper record, control responses to cummulative concentrations of tyramine (T) (1 X 10^{-7}, 1 X 10^{-6}, 1 X 10^{-5}) (dots), followed by oil immersion (O). Lower record, control response to noradrenaline (NA) (1 X 10^{-8}), followed by oil immersion.

b. Responses of strips pretreated with iproniazid and then treated as in a. Tyramine contracted strip was returned to Krebs solution at (K). All 4 strips were from the same aorta.

aortic strips in Krebs solution, and this was unaffected by inhibition

of MAO. The responses of 8 control and of 8 iproniazid pretreated

strips cut from the same aortas were 3.4 ± 0.8 and 3.8 ± 0.8 mm,

respectively. However, the contractions of strips exposed to bretylium

increased markedly after oil immersion (Fig. 30a, #1). Bretylium is

not a substrate for MAO, and iproniazid pretreatment did not affect

the behavior of these strips in oil.

Evidence that the contraction of aortic strips by bretylium

involves receptors which are blocked by phenoxybenzamine (POB) is shown

in figure 30a, #2. Pretreatment with POB (1×10^{-7}) for 10 minutes

completely eliminated the response to bretylium (1×10^{-4}) both before

and after oil immersion. Subsequent responses to KCl demonstrated that

this and the control strip cut from the same aorta were comparably

reactive.

More direct evidence that the progressive contraction in oil

of strips exposed to bretylium or tyramine was due to the release of

endogenous noradrenaline was obtained in experiments with aortic strips

from reserpine pretreated rabbits. Reserpine is known to deplete

tissue stores of catecholamines (See Historical Introduction.) and to

depress responses to tyramine and other agents which are believed to

act through the release of endogenous amine. Aortic strips from

reserpinized rabbits responded to bretylium (1×10^{-4}) with only a

slow contraction of limited amplitude after oil immersion (Fig. 30b).

Aortic strips from reserpinized animals pretreated with iproniazid

responded to the cumulative addition of tyramine (1×10^{-5}, 1×10^{-4}),

but showed only a very minor, slow progression of the contraction after
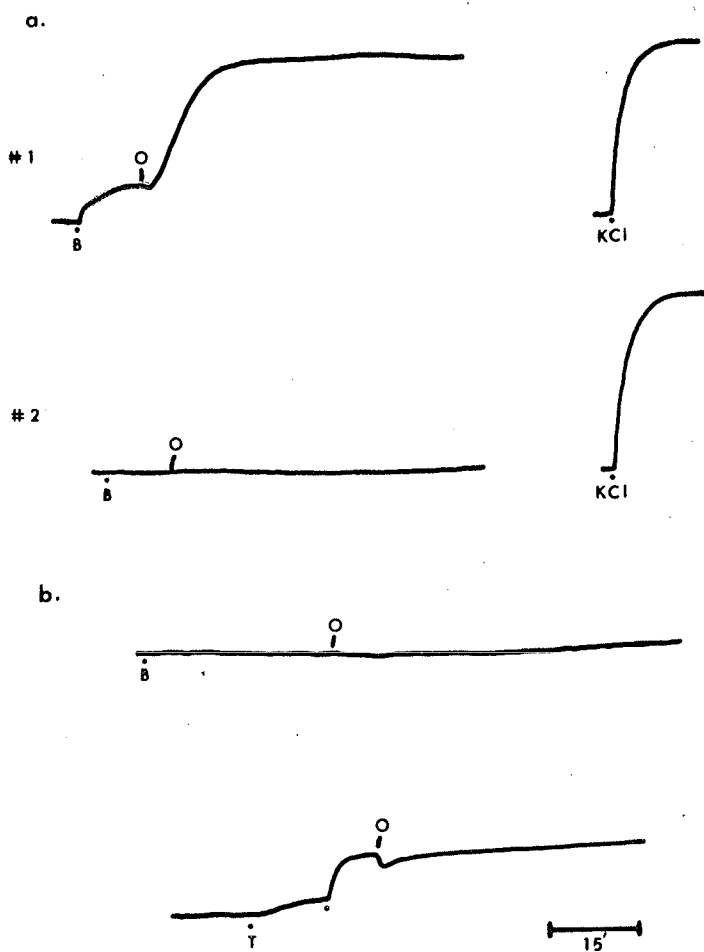
Fig. 30.   Responses of Unreserpinized and Reserpinized Aortic Strips to Bretylium and Tyramine.

a.   Left, responses of 2 unreserpinized strips to bretylium (B) (1×10^{-4}), followed by oil immersion (O); strip #2 was pretreated with phenoxybenzamine (1×10^{-7} for 10 min.). Right, responses of the same strips to KCl (0.07 M).   b.   Responses of reserpinized strips to bretylium (upper), and to high cumulative concentrations of tyramine (T) (1×10^{-5} and 1×10^{-4}) (lower), both followed by oil immersion.   The strip exposed to tyramine was pretreated with iproniazid.

oil immersion (Fig. 30b). The initial response to tyramine probably was due to a direct action of this sympathomimetic rather than to release of endogenous catecholamines (Furchgott, 1955). The minimal further contraction after oil immersion indicates that only minute amounts of endogenous amine were released.

Although pretreatment with reserpine drastically and consistently reduced the progressive contraction in oil of strips treated with tyramine or bretylium, this component of the response was never completely absent and, on occasion, was considerable greater than that shown in figure 30b. The magnifying effect of oil immersion, attributable to trapping all of the released catecholamine within the tissue, was apparently adequate to reveal otherwise undetectable rates of endogenous catecholamine release. The extreme sensitivity of this technique in detecting the release of catecholamines was further demonstrated by the responses of strips treated with a combination of iproniazid, tropolone and cocaine.

Strips pretreated with iproniazid and subsequently treated with tropolone (3×10^{-5}) for 30 minutes and then with cocaine (1×10^{-5}) for 10 minutes gradually contracted after oil immersion. The mean amplitude of responses of 11 strips from 4 aortas was 2.0 mm after 5, 8.1 mm after 15 and 12.8 mm after 30 minutes of oil immersion. Strips from only 1 of the 4 aortas showed any detectable contraction before oil immersion. When these are excluded, the mean amplitudes of contraction of 8 strips were 0.4 mm after 5, 3.8 mm after 15 and 8.5 mm after 30 minutes of oil immersion. The combination of iproniazid, tropolone and cocaine produced no contraction of strips from reserpine

pretreated animals in either Krebs solution or oil, confirming that

endogenous noradrenaline was involved in the response. The slow

contraction observed probably was due to "spontaneously" released

catecholamine which was protected from enzymatic degradation and other

inactivation processes by the combined effects of iproniazid, tropolone

and cocaine, and from dissipation into the surrounding medium by the

oil immersion. It is clear that the oil immersion technique can reveal

the presence of free catecholamine even when the amounts released are

well below those required to produce any response when the tissue is

in an aqueous medium.

The release of endogenous noradrenaline demonstrated in

strips treated with iproniazid, tropolone and cocaine probably did not

alter the course of relaxation in oil of strips contracted by noradren-

aline or adrenaline after exposure to these inhibitors. The mean

contraction amplitude recorded after 30 minutes of oil immersion was

approximately equivalent to that produced by a concentration of

1×10^{-9} noradrenaline. This would be insufficient to alter the minor

relaxation in oil of noradrenaline (1×10^{-8}) contracted strips treated

with the 3 inhibitors of catecholamine inactivation. This is confirmed

by the observation that the relaxation of strips from reserpinized

animals after contraction by noradrenaline (1×10^{-8}) was not detectably

different from that of unreserpinized strips.

2.   Effect of Incubation with Noradrenaline on Responses to Bretylium:

Many investigators have reported that poor responses to

tyramine in a variety of preparations can be augmented by prior

infusion of noradrenaline (See Historical Introduction.) Furchgott

(personal communication, 1965) found that treatment of aortic strips from reserpinized animals with iproniazid before "priming" with noradrenaline, increased its effect on subsequent responses to tyramine.

Incidental observations during studies with bretylium suggested that preincubation with noradrenaline augmented responses to this agent, even those of strips from unreserpinized animals. This was further investigated using pairs of strips from the same aortas. One of each pair was pretreated with iproniazid. The paired strips were "primed" by exposing them for varying periods of time to noradrenaline (3×10^{-9} to 1×10^{-6}), followed by washout and relaxation to basal tone. The mean contractions of the control and iproniazid pretreated strips in response to bretylium (1×10^{-4}) were 0.6 ± 0.4 mm and 4.4 ± 1.2 mm, respectively. This difference is significant at the 1% level of probability.

3.   Behavior in Oil of Aortic Strips Exposed to Guanethidine:

Maxwell et al. (1962) reported that the response of aortic strips to guanethidine was highly variable. This was confirmed in the present studies. Small contractions were occasionally produced by guanethidine, in concentrations of from 1×10^{-5} to 3×10^{-5}, but very often there was no detectable response of aortic strips in Krebs solution.

However, strips exposed to guanethidine (3×10^{-5}) consistently contracted after oil immersion. This slow response was markedly reduced in magnitude in strips from reserpinized animals and was eliminated by pretreatment with phenoxybenzamine (1×10^{-7}) for

10 minutes.

Pretreatment of aortic strips with iproniazid potentiated the response to guanethidine (not itself a substrate for MAO) in Krebs solution. The response of 8 control strips to guanethidine. (3 X 10^{-5}) was 0.38 $\pm$ 0.2 mm and that of 8 iproniazid pretreated strips from the same aortas, 4.38 $\pm$ 0.9 mm. This difference is significant at the 0.1% level of probability. Iproniazid pretreated strips which had contracted in response to guanethidine during oil immersion, relaxed promptly when returned to Krebs solution. However, repeated contractions, of progressively diminishing magnitude, could be obtained by reimmersing the strips in oil without re-exposure to guanethidine (Fig. 31). Recontraction also occurred, but was considerably less prominent, in strips not pretreated with iproniazid. These results indicate that the catecholamine releasing action of guanethidine is not readily terminated by "washing out" the drug.

G. ANCILLARY EXPERIMENTS - RELAXATION OF 5-HYDROXYTRYPTAMINE AND HISTAMINE CONTRACTED STRIPS IN OIL

Preliminary investigations were made of the behavior in oil of aortic strips contracted by 5-hydroxytryptamine (5-HT) and histamine to assess the presence of endogenous mechanisms for the inactivation of nonsympathomimetic amines in aortic tissue. The sensitivity of strips cut from different aortas to 5-HT varied widely, and this preliminary study was limited to the question of whether 5-HT, a known substrate for MAO (Blaschko, 1952), is indeed, inactivated by
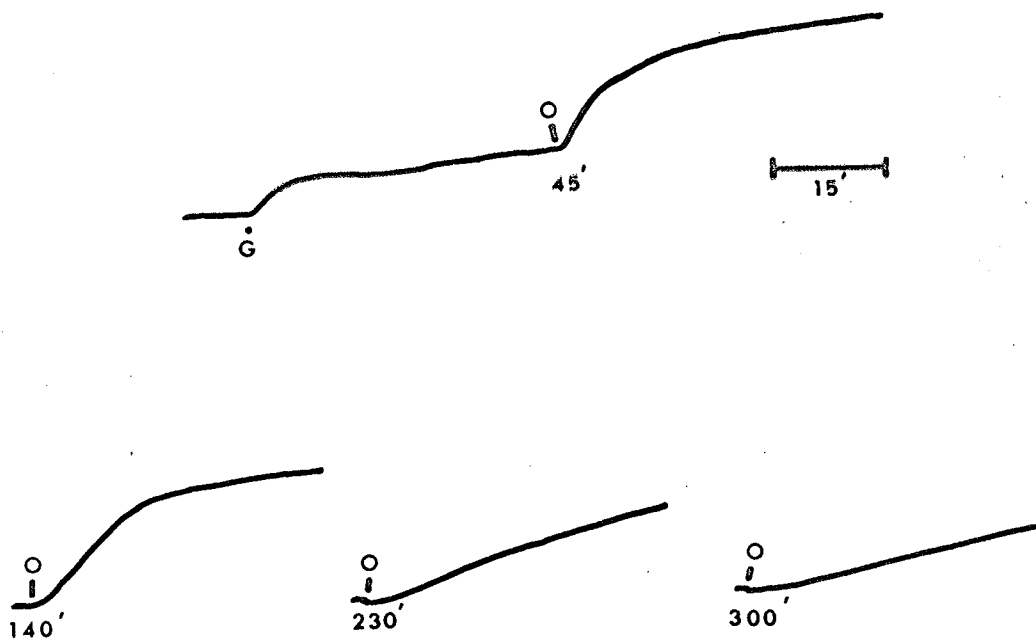
Fig. 31. Behavior of Guanethidine Treated Aortic Strip in Oil.

Upper, response of an iproniazid pretreated strip to guanethidine (G) (3 X 10^{-5}), followed by oil immersion (O). The strip was then returned to Krebs solution and allowed to relax. The lower records show the effects of repeated oil immersions, separated by relaxation in Krebs solution, at the indicated intervals after the single exposure to guanethidine.

this enzyme in aortic tissue. Vane (1959) concluded that 5-HT does not

reach the site of its potential deamination in smooth muscle because

inhibitors of MAO potentiated responses of the rat stomach strip to

tryptamine but not those to 5-HT, although the MAO in ground rat stomach

tissue deaminated both at comparable rates.

A number of experiments comparing responses of iproniazid

pretreated and control strips to concentrations of 5-HT ranging from

3×10^{-8} to 1×10^{-6} clearly demonstrated that deamination is an

important mechanism terminating the action of this compound in aortic

tissue. The results of a representative experiment are shown in

figure 32a. Two strips from a single aorta, one pretreated with

iproniazid, were contracted by 5-HT (1×10^{-7}), and their subsequent

relaxation in oil recorded. The much delayed relaxation of the

iproniazid pretreated strip indicated that MAO plays a major role in

the inactivation of 5-HT in rabbit aortic tissue. A few of the control

strips contracted with 5-HT relaxed only very slowly in oil. No

explanation of this observation can be offered at the present time.

Recent work by Snyder and Axelrod (1965) indicated 2 potential

pathways of metabolic inactivation for histamine: deamination by

diamine oxidase and methylation by histamine-N-methyl transferase.

Aortic strips contracted with histamine were found to relax quite

promptly in oil and a preliminary attempt was made to identify routes

of endogenous inactivation of this amine. Two strips were pretreated

with semicarbazide (1×10^{-6}), an effective inhibitor of diamine

oxidase (Zeller, 1956), for about 30 minutes and, without washout,

were contracted by the cumulative addition of histamine (3×10^{-8},

a.

b.
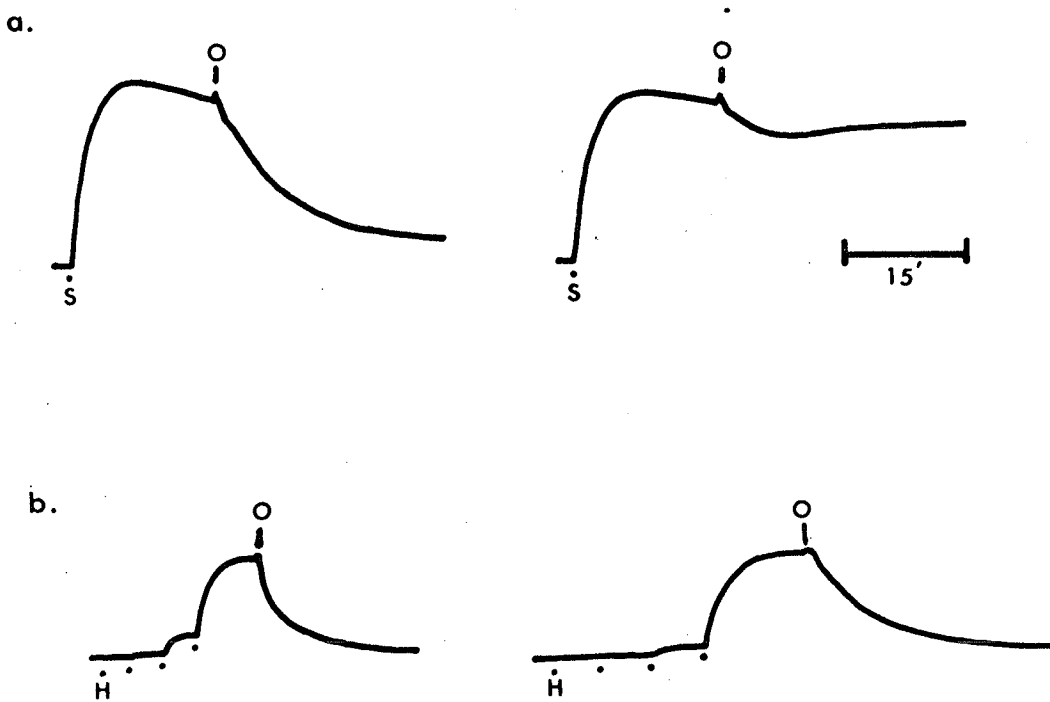
Fig. 32. Effects of Enzyme Inhibitors on Responses of Aortic
Strips to Histamine and 5-Hydroxytryptamine and Relaxation after
Oil Immersion.

a. Responses of 2 strips from the same aorta to
5-hydroxytryptamine (S) (1×10^{-7}), followed by oil immersion (O).
Left, control strip; right, iproniazid pretreated strip. b. Re-
sponses of 2 strips from the same aorta to cumulative concentra-
tions of histamine (H) (3×10^{-8}, 1 and 3×10^{-7}, 1×10^{-6}) (dots),
followed by oil immersion. Left, control strip; right, semicarbazide
(1×10^{-6}) pretreated strip.

1 and 3 X 10^{-7}, 1 X 10^{-6}). Their subsequent relaxation in oil was

compared to that of control strips from the same aortas and found to

be decreased (Fig. 32b and Fig. 33). Incubation of one strip with

semicarbazide (1 X 10^{-5}) reduced the rate of relaxation no more than

did a concentration of 1 X 10^{-6}. In one experiment iproniazid, which

also effectively inhibits diamine oxidase (Zeller, 1956), slowed the

relaxation of a histamine contracted strip in oil, and the addition

of semicarbazide to an iproniazid pretreated strip had no additional

effect on the rate of relaxation.

These results suggest that diamine oxidase plays a signifi-

cant role in the inactivation of histamine in aortic tissue, and that

at least one other pathway, perhaps histamine-N-methyl transferase, is

also of importance.
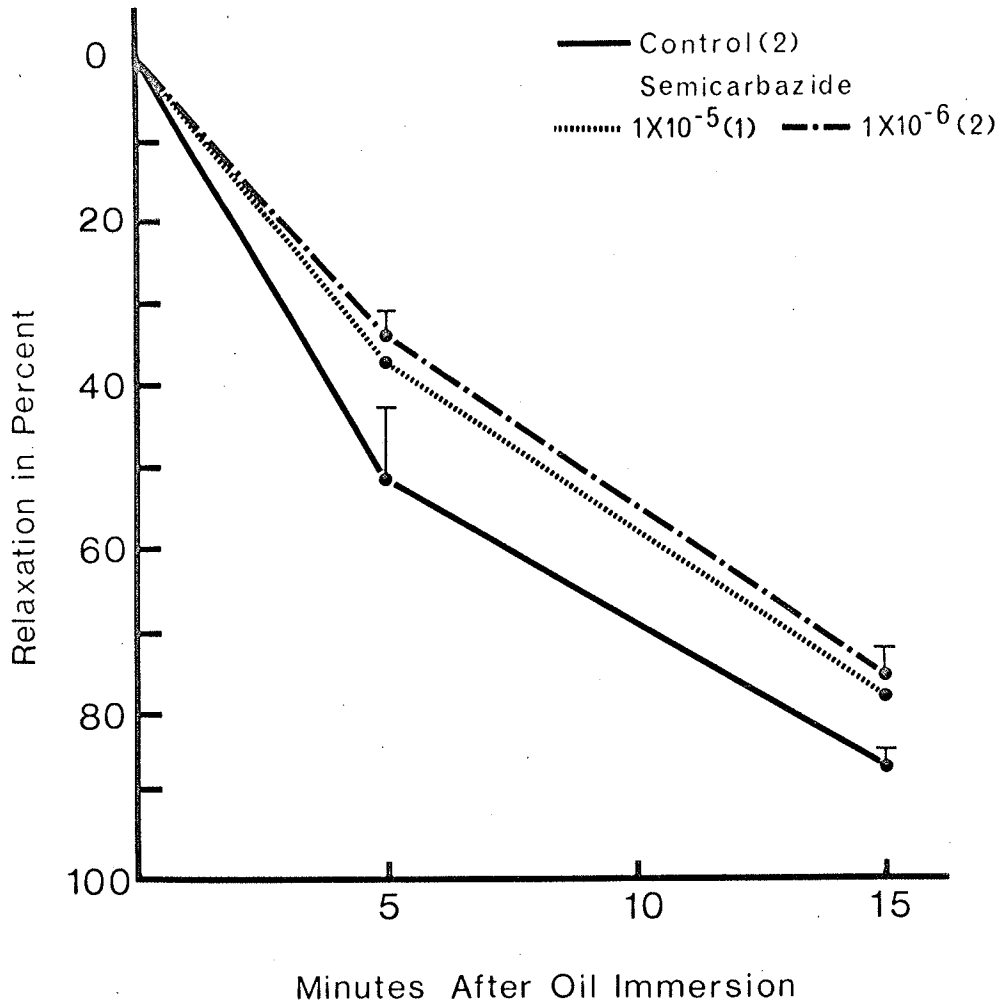
Fig. 33. Effect of Semicarbazide on the Relaxation of Histamine
Contracted Aortic Strips after Oil Immersion.

Figures in parentheses indicate number of preparations
represented by each curve. Bars indicate standard errors of means.
All strips were contracted by histamine (3×10^{-6}).
Statistical analysis:

Min.

Semicarbazide (1×10^{-6} and
1×10^{-5}) vs. Control

$5 - P < 0.2$
$15 - P < 0.05$

H. DISCUSSION - THE DISPOSITION OF SYMPATHOMIMETIC AMINES

The recent availability of tritiated catecholamines with high specific activity and concomitant improvements in analytical techniques for the identification and separation of these amines and their metabolites has produced a plethora of interest in the disposition of these neurohumors in various organs and tissues and its relationship to their physiological and pharmacological effects. However, the preparations studied have not accurately reflected the range of adrenergically regulated systems. Because of the striking capacity of the heart to exchange, accumulate and store catecholamine (Raab and Gigee, 1955; Stromblad and Nickerson, 1961; Whitby et al., 1961; Westfall, 1965, and many others), investigation of the roles of various inactivation mechanisms in adrenergic function has been focused on this organ. The proficiency of the heart in extracting circulating noradrenaline is typified by the finding that 56% of the dl-H^3-noradrenaline perfused through dog hearts at a rate of about 0.3 μg/min. for 10 minutes was retained in the organ (Potter et al., 1965). In the opinion of the authors this may reflect nearly complete extraction of the l isomer. It has even been suggested that uptake from the circulation could be a physiological means of supplying neurotransmitter to this organ (Axelrod, 1965).

Unfortunately, assessments of the relative importance of various mechanisms in the inactivation of noradrenaline and related amines derived almost exclusively from observations on the heart have been extended, without sufficient confirmation, to encompass the panoramic diversity of sympathetic effector systems, which are predominantly smooth muscle and glands. Studies on smooth muscle

structures per se (e.g., Hertting and Axelrod, 1961; Rosell et al., 1963)

have been much less frequent and more limited in scope than those on the

heart.

A few pertinent studies have dealt peripherally or directly

with routes of metabolic inactivation for catecholamines in vascular

smooth muscle. Whitby et al. (1961) assayed a variety of cat tissues

for H^3-noradrenaline and a single metabolite, normetanephrine, up to 2

hours after the intravenous injection of 25 ug/kg of the former, and

found concentrations of both in the aorta greater than those in the

blood. Rosell et al., (1963) found that a perfused skeletal muscle

vascular bed of the cat spontaneously released both O-methylated and

deaminated, O-methylated metabolites of previously administered H^3-nora-

drenaline. The results of both studies suggested an important role for

catechol-O-methyl transferase (COMT) in the metabolic inactivation of

noradrenaline in vascular smooth muscle.

The assessments of routes of amine inactivation in vascular

smooth muscle presented in this thesis are based on determinations of

the dissipation of the effects of various sympathomimetic amines with

time rather than on the more usual analytical procedures for the deter-

mination of the amines and their metabolites in tissues. This approach

required 1) demonstration that the residual response is dependent on

residual active amine in the vicinity of tissue receptors (Section III, A),

2) elimination of dissipation of amine by diffusion into the medium sur-

rounding the isolated smooth muscle preparation under study (accomplished

by the technique of oil immersion, Section III, B), and 3) the use of

drugs to inhibit specific pathways of inactivation. Such agents rarely

have absolute specificity, but the agents employed were checked in a
variety of ways to detect ancillary actions which might affect inter-
pretation of the results. Where evidence that an inhibitor (e.g.,
cocaine) affected more than one process was obtained, special studies
were undertaken in attempt to assess the relative contribution of each
to the observed effects.

In addition to establishing that relaxation of amine contracted
aortic strips in oil reflected declining concentrations of agonist in
the tissues, it was necessary to provide a basis for relating these two
quantitatively. This was done by equating the percent residual contrac-
tion at any time during relaxation with the concentration of drug re-
quired to produce a response equal to the same fraction of that produced
by the initial concentration of agonist. This concentration was obtained
from separately determined dose-response curves.

Such transformations were particularly important in comparing
different agonists and the behavior of reserpinized and unreserpinized
preparations.

The concentration of 1×10^{-8} noradrenaline or adrenaline used
in many of the studies reported in this section probably gives a reasona-
ble approximation of physiological tissue levels of amine. This was
indicated by the fact that it produced a roughly half maximal contrac-
tion, which may reasonably be equated with the tone of vascular smooth
muscle maintained by sympathetic nerve activity.

Studies with this concentration of catecholamine indicated
that MAO makes little contribution to the inactivation of either adre-
naline or noradrenaline. The finding that iproniazid in a concentration

which insured virtually complete inhibition of MAO did not alter the ability of strips to inactivate these amines is in agreement with the results of others using different techniques and tissues (e.g., Hertting et al., 1961b; Brown and Gillespie, 1957, Potter et al., 1965). However, it was found in the present experiments that inhibition of COMT significantly slowed the relaxation in oil of noradrenaline and, particularly, of adrenaline contracted strips, the period required for half relaxation being increased to 1.3 and 2.9 times those of controls, respectively. This is in agreement with the conclusion of Rosell et al. (1963) that O-methylation is the major initial enzymatic process for the inactivation of noradrenaline in vascular smooth muscle of the cat in situ.

The result of inhibiting any particular endogenous pathway which might contribute to the inactivation (termination of action) of a given sympathomimetic amine has been expressed in terms of the time required for half relaxation, relative to that of the appropriate control preparation, i.e., changes in the time for 50% relaxation from 3.5 to 7.0 and from 10 to 20 minutes are both expressed as "2.0 times the control value". Provided a steady state has been achieved prior to oil immersion, these values can be converted quantitatively to an expression of the residual capacity of the strips to inactivate amine, the reciprocal of the decrease due to the treatment being investigated, by simply dividing the total capacity (100%) by the shift in time required for 50%, or some other convenient fraction, relaxation. Thus, the time for 50% relaxation in oil after contractions produced by adrenaline (1×10^{-8}) was 4.2 and 12.2 minutes for control and tropolone pretreated strips, respectively, which represented a shift to 2.9 times the control value,

and indicated that at least 65% of this low concentration of adrenaline was *in* activated by O-methylation in aortic tissue (12.2/4.2 = 2.9; 100/2.9 = 35% residual, or 65% inhibited). Similarly, the observed increase in the relaxation time of strips contracted by noradrenaline (1 X 10^{-8}) indiacted that at least 25% of this amine was inactivated by the same enzyme. Thus, although adrenaline is the more effectively O-methylated, COMT appears to function as the major initial enzymatic inactivation pathway in aortic tissue for both of these catecholamines.

Inhibition of both MAO and COMT increased the time required for 50% relaxation of strips contracted with noradrenaline (1 X 10^{-8}) to about 3.2 times that of controls, indicating that the aortic tissue normally inactivates almost 70% of this amine enzymatically and the shift to about 6.0 times the control relaxation time observed under the same conditions with adrenaline indicated that slightly over 80% of this amine is handled by the same processes. Although it could not be shown to contribute to their disposition in the presence of normal COMT activity, these results indicate that MAO functions as an important alternate mechanism for the inactivation of both amines. Indeed, the addition of iproniazid shifted the intervals required for 50% relaxation to 2.4 and 2.1 times those of strips treated with tropolone only for noradrenaline and adrenaline, respectively, indicating that in the absence of COMT, MAO accounts for over half of the total amounts of both of these amines handled by endogenous mechanisms.

To reconcile the results obtained with each enzyme inhibitor alone with the observed effects of the combination, i. e., the apparent absence of any contribution of MAO to the normal inactivation of

noradrenaline and adrenaline and its important contribution after inhi-
bition of COMT, it appears necessary to postulate that access to these
two enzymes by the catecholamines is unequal. The results suggest that
COMT and MAO function in "series", and it is quite possible that this
behavior has a morphological basis. COMT appears to be primarily, if
not entirely, a soluble, cytoplasmic enzyme (Axelrod, 1959a, 1965; Alberi-
ci et al., 1965), whereas MAO is predominantly a mitochondrial enzyme,
although some activity has been found in "microsomal" fractions of cells
(Baudhuin et al., 1964). These distributions could explain the apparent
series function in that catecholamine penetrating a cell to reach intra-
cellular loci of inactivation would have to pass through a field of COMT
activity before reaching a mitochondrial, or other particulate, site of
deamination. Thus, the effectiveness of an alternate pathway can lead
to underestimation of the contribution of a major mechanism of inactiva-
tion when only the latter is inhibited. In the present experiments, the
effect of inhibiting both COMT AND MAO indicated that about 70% of this
concentration of noradrenaline was inactivated enzymatically, and since
inhibition of MAO alone did not alter the rate at which aortic strips in-
activated noradrenaline, there appears to be a reasonable basis for sug-
gesting that the proportion of this amine normally inactivated by primary
O-methylation is closer to 70 than to 25% of that handled by all mecha-
nisms of disposition in aortic tissue. The major capacity of MAO to
function as a primary inactivation pathway, although it normally appears
to act on an already inactive molecule, normetanephrine, could well have
contributed to the failure of previous studies to show an effect of
COMT inhibition on responses to noradrenaline despite the normally domi-
nant role of this enzyme in its metabolic inactivation.

The picture with respect to termination of the action of adrenaline is somewhat less complicated than that of noradrenaline. The decrease in the rate of inactivation after dual enzyme inhibition indicated a contribution of metabolism to inactivation of adrenaline which was not far from that obtained from the sum of the effects of each inhibitor alone. The decrease in rate of relaxation when COMT and MAO were simultaneously inhibited showed that about 80% of the adrenaline (1×10^{-8}) was enzymatically inactivated, whereas the sum of the effects of the individual inhibitors indicated about 65 to 70%.

The experiments discussed in this section demonstrated that O-methylation is the major metabolic pathway for the inactivation of both noradrenaline and adrenaline in aortic tissue. However, the role of COMT in the metabolism of adrenaline was much more obvious than in the case of noradrenaline. There is no direct evidence that the affinity of catechol-O-methyl transferase for these two amines differs appreciably. The results of Axelrod and his associates (Axelrod et al., 1959; Whitby et al., 1961) demonstrating that during the first 5 minutes after the injection of tritiated amine about 70% of adrenaline and less than 50% of noradrenaline was O-methylated could be explained on the basis of a greater affinity of adrenaline for the transferase. However, they chose to interpret these results as reflecting a greater binding of noradrenaline, which more effectively protected it from enzymatic metabolism. (See Axelrod, 1964.)

The much smaller effect of inhibiting COMT alone on the inactivation of noradrenaline appears to represent a greater ability of MAO to take over as the primary inactivating enzyme than in the case of adrenaline. This may be due to a greater affinity of MAO for the

noradrenaline. Blaschko (1952) reported that noradrenaline was deaminated more rapidly than was adrenaline by a rabbit liver preparation of MAO presented with an equimolar mixture of the two amines.

In summary, it appears that, under various experimental conditions, the morphological distribution of COMT and MAO plus a greater affinity of MAO for noradrenaline than for adrenaline could considerably magnify what is normally a relatively small disparity in the extent to which primary O-methylation contributes to their disposition.

The observations on enzymatic inactivation of 100 times higher concentrations of noradrenaline and adrenaline may be interpreted on the basis of the same combination of factors required to account for the characteristics of the inactivation of concentrations much closer to the physiological, and appear to confirm them. Changes in the times for half relaxation after inhibition of MAO or COMT indicated that at amine concentrations of 1×10^{-6}, MAO was responsible for the inactivation of 60 to 65% of the noradrenaline and about 40% of the adrenaline, and the transferase for about 15 to 20% and 60 to 65%, respectively. Thus at this concentration the predominant metabolic pathway for the inactivation of noradrenaline is deamination and that of adrenaline is O-methylation, with MAO playing a lesser, but still considerable, role. This high concentration of amine might be expected to swamp any organized, sequential system for handling physiological amounts and result in the simultaneous presentation of intact amine to both enzymes. In this situation enzyme inhibition could show the true capabilities of the two enzymes to inactivate noradrenaline and adrenaline, and they did, in fact, reveal a situation very similar to that deduced from an analysis of the results of the more complex studies of inhibitors required with

the low concentrations of catecholamines. The results reported by Trendelenburg (1965) can be interpreted as showing similar enzymatic components for the transhepatic inactivation of large doses of catecholamines.

An important role of MAO in the inactivation of a high concentration of noradrenaline (1 X 10^{-5}) was also apparent in preliminary studies (Section III, A), where it was found that iproniazid slowed the relaxation of aortic strips even in an aqueous medium, a much less sensitive and quantitative experimental situation than oil immersion. Although MAO was shown not to have a primary role in the inactivation of noradrenaline and adrenaline except at high concentrations of the amines, this enzyme clearly functioned in the inactivation of moderate concentrations of phenylephrine. The time required for 50% relaxation of strips contracted with phenylephrine (3 X 10^{-8}) was increased to greater than 9 times that of the controls by iproniazid, indicating that close to 90% was inactivated by deamination. There appeared to be no other pathways for the metabolic inactivation of phenylephrine in aortic tissue, and a limiting rate of deamination may account for the fact that untreated control strips inactivated phenylephrine at only half the rate for adrenaline or noradrenaline.

To relate the metabolic disposition of amines, discussed above, to other endogenous processes, it is necessary to consider the location of the enzymes involved. The deaminated metabolites of catecholamine found in sympathetically innervated organs are widely regarded to reflect almost exclusively intraneuronal metabolism (Belleau et al., 1961; Axelrod, 1965; Brodie and Beaven, 1963; Kopin, 1964). The ori-

gin of this view is not entirely clear, but its roots are probably in the early observation that the large amounts of endogenous catecholamine released by reserpine produce negligible sympathomimetic effects if MAO has not been inhibited. Indeed, the effect of MAO inhibition on the early response to reserpine was noted even before it was generally recognized that this agent causes the release of catecholamines (Chessin et al., 1957). Acceptance of the predominant importance of intraneuronal MAO was solidified by the writings of many workers, who found, with the aid of tritiated catecholamines, that primary deamination was not a major fate of either injected amine or of that released by nerve impulses, but that catecholamine released by reserpine from endogenous stores was predominantly deaminated.

Although involvement of intraneuronal MAO in determining the characteristics of certain adrenergic effects cannot be denied, both an analysis of the available literature and the present findings indicate that the bulk of the MAO activity is in non-nervous tissue, predominantly smooth muscle in the aortic strip preparation. Koelle and Valk (1954) provided direct histochemical evidence of the localization in smooth muscle of most of the MAO in the walls of blood vessels. They found most of the staining, indicative of MAO, to be in the media of the several different types of vessels studies. The media of large arteries of the rabbit was more heavily stained than were the arterioles, despite the considerably greater sympathetic innervation of the small vessels and the fact that the adrenergic ground plexus of blood vessels lies external to and not within the media. MAO was also found in sympathetic nerves, but the concentration appears not to be remarkably greater than that in many other types of cells, and nerve fibers constitute only

a minute fraction of the cellular mass of effector organs.

Other evidence that MAO is predominantly an effector cell rather than an intraneuronal enzyme is the report that chronic sympathetic denervation of the rabbit's ear does not significantly alter the MAO activity of its arteries (Armin et al., 1953), and the minimal effect of transplantation on the MAO content of dog hearts. Potter et al. (1965) completely denervated dog hearts by transplantation and found right atrial, left ventricular and interventricular septal MAO to be unaltered and that of the right ventricle and left atrium to be only slightly reduced. The formation of deaminated metabolites of infused noradrenaline was undiminished by the denervation procedure, indicating that the enzyme measured in vitro was effective in situ. MAO is also found in uninnervated placenta in amounts comparable to those in spleen, veins, adrenals and brain (Thompson and Tickner, 1949; Davison, 1958).

An extraneuronal site of the MAO activity observed in the present experiments was indicated by the finding that iproniazid produced a marked further reduction in the rate of inactivation of both noradrenaline and adrenaline when added to the combination of tropolone and cocaine, the latter in a concentration which produced almost the maximal effect of this agent on amine inactivation, and which was undoubtedly effective in blocking most of the transport of catecholamine into nerves.

The results of studies which will be presented and discussed in the next section of this thesis, showed the effect of iproniazid to be essentially the same in aortic strips from reserpinized and unreserpinized rabbits. It is widely accepted that inhibition of incorporation

into storage granules by reserpine leads to the intraneuronal deamina-
tion of catecholamines transported across the nerve cell membrane
(Axelrod, 1965; Kopin and Gordon, 1963; Kopin, 1964; Stjärne, 1964;
Malmfors, 1965; Potter et al., 1965). The failure of reserpinization
to cause an obvious increase in the contribution of deamination to the
inactivation of catecholamines in aortic tissue probably reflects the
very limited contribution of intraneuronal enzyme to the total effective
MAO in this vascular smooth muscle preparation.

Despite the dominance of extraneuronal MAO, some involvement
of intraneuronal enzyme was suggested by the results presented in this
section with agents such as bretylium and guanethidine, which are repu-
ted to stimulate smooth muscle through the release of endogenous cate-
cholamines. Responses to guanethidine, but not those to bretylium,
were potentiated by pretreatment with iproniazid. Neither of these
agents is itself a substrate for MAO, and the most plausible explana-
tion for the observed difference appears to be differences in the intra-
cellular routes traversed by the released catecholamine, one in close
proximity to and the other relatively isolated from loci of MAO activity.
These routes might be associated with different components of the intra-
neuronal store of catecholamine. However, although amine released by
bretylium appears to be largely unaffected by intraneuronal MAO, it was
demonstrated that a route rich in MAO must be traversed by exogenous
noradrenaline to reach the storage sites from which it is released by
bretylium. Responses to bretylium were markedly greater in strips pre-
incubated with noradrenaline after exposure to iproniazid than in strips
treated in the same way except for exposure to the enzyme inhibitor.

In summary, it appears that catecholamines released from endo-
genous stores by some agents, particularly reserpine, may be affected
by intraneuronal MAO before reaching effector cells and that that MAO
may also act on noradrenaline passing to storage sites in adrenergic
nerves. However, there is no evidence to indicate that intraneuronal
MAO is involved in terminating the action of sympathomimetic amines,
either exogenous amine, as administered in the present experiments, or
noradrenaline after its release by nerve impulses. By far the major
part of the MAO in most organs is extraneuronal, in effector cells, and
the effect of iproniazid in the presence of cocaine, reported above,
indicated that it is this extraneuronal enzyme which is involved in
situations in which MAO participates in terminating the action of sympa-
thomimetic amines.

There appears to be general agreement that O-methylation occurs
extraneuronally, in effector cells. Evidence for this site is well
illustrated by the report that COMT activities in all parts of dog
hearts were unaltered after complete denervation by transplantation
(Potter et al., 1965). In contrast to MAO, there is no evidence that
inhibition of COMT alters the effects of endogenous catecholamine released
by drugs.

Enzymatic processes are only one facet of the inactivation of
sympathomimetic amines in tissues, and they can be properly assessed
only in relation to the contributions of other mechanisms. As indi-
cated in the Historical Introduction, the concept of inactivation of
amines by uptake and storage in various effector organs has rapidly
grown to assume a dominant position in the field of adrenergic mecha-
nisms and now provides the standard explanation for a wide variety
of physiological and pharmacological phenomena. Observations supporting

the importance of this mechanism have come largely from studies on the uptake and retention of tritiated amine, presumably mostly by adrenergic nerves within the effector organs involved. That such uptake does occur and that it can be inhibited by cocaine and certain other drugs is unquestioned, but the importance of its contribution to the overall process of amine inactivation is much less clear. Acceptance of simple uptake and retention of labelled amine as a measure of the role of this mechanism in inactivation or termination of action has been strongly criticised on the basis that the tracer technique cannot distinguish between net uptake and exchange (Strömblad and Nickerson, 1961; Nickerson, 1965). It is clear that both processes can occur, but only the former could make any contribution to inactivation. Consequently, unless information on changes in total amine content is also provided, which is rarely the case, data obtained by the tracer uptake techniques may grossly overestimate the importance of this process in termination of action. Certainly current dynamic concepts of the adrenergic-neuroeffector junction, as reflected in models which assign an important role in amine inactivation to nerve uptake (Brodie and Beaven, 1963; Kopin, 1964; Koelle, 1965), are sensitive to such an artifact.

The results presented in this section provide information on non-enzymatic as well as on enzymatic mechanisms of sympathomimetic amine inactivation, and of the interrelationship of the two. Cocaine was found to inhibit the rate of inactivation of noradrenaline, adrenaline and phenylephrine by strips of rabbit thoracic aorta. Treatment with cocaine (1×10^{-5}) increased the time required for 50% relaxation of strips contracted by 1×10^{-8} noradrenaline or adrenaline to about 1.5 times of the controls, indicating that the mechanism interfered

with by cocaine was responsible for the inactivation of about 35% of each amine. Cocaine had a similar effect on inactivation when added to treatment by iproniazid or iproniazid plus tropolone, indicating that the cocaine sensitive mechanism is largely independent of MAO and COMT activity.

Cocaine (1×10^{-5}) slowed the relaxation of strips contracted by phenylephrine (3×10^{-8}), and when combined with iproniazid pretreatment almost completely prevented inactivation of the amine. However, these results suggested that the actions of cocaine and of MAO inhibition were not completely independent. The increase in the time required for 20% relaxation (50% was not reached during the 30 minute observation period) produced by iproniazid pretreatment alone indicated that MAO was responsible for the inactivation of 85 to 90% of the phenylephrine, and the decrease in the rate of relaxation produced by cocaine alone indicated that the cocaine sensitive mechanism accounted for 40 to 45% of the capacity to inactivate the amine. There are several possible explanations for this finding: a) Cocaine in a concentration of 1×10^{-5} inhibits MAO. b) Part of the deamination of phenylephrine is intraneuronal, and cocaine, by blocking nerve membrane transport, prevents access of amine to this portion of the enzyme, c) Cocaine prevents access of amine to MAO in effector cells.

It appears most unlikely that cocaine directly inhibited MAO in these experiments. Prolonged exposure to a 10 times higher concentration of the much more powerful MAO inhibitor, iproniazid, was required to assure inhibition in aorta strips, and the concentration of cocaine used was less than 1/500th that which Philpot (1940) found necessary to inhibit the MAO of liver homogenates by 80%.

Cocaine was shown to impair significantly the inactivation of

phenylephrine by aortic strips even after MAO inhibition, indicating

that at least a portion of its effect was due to interference with

binding and storage, presumably inhibition of membrane transport of

the amine. This action could prevent access to intraneuronal MAO,

and if this were involved in the inactivation of phenylephrine, might

explain the observed overlap. However, this explanation requires cer-

tain specific conditions, which could not be demonstrated in other ex-

periments. It would be applicable only if deamination were a suffi-

ciently important component of the fate of amine entering nerves so

that after MAO inhibition binding and other intraneuronal mechanisms

could not handle the transported phenylephrine. Under these circum-

stances, back diffusion could reduce net transport and produce the

observed effect. However, it was found that cocaine slowed the inac-

tivation of phenylephrine by aortic strips from reserpinized animals

pretreated with iproniazid in a manner similar to its effect in unreserpi-

nized preparations pretreated with the MAO inhibitor. Since the combi-

nation of reserpine and iproniazid should have eliminated both mechanisms

for intraneuronal disposition of phenylephrine, transport should have

reached equilibrium with back diffusion during the relatively prolonged

exposure to phenylephrine before exposure to cocaine and oil immersion,

and the cocaine should have added nothing to the slowing of relaxation

produced by iproniazid.

The finding that MAO inhibition and cocaine decreased the

capacity of aortic strips to inactivate phenylephrine by 85 to 90% and

40 to 45%, respectively, appears to be most satisfactorily explained

on the assumption that, in addition to its action on nerves, cocaine

acts on effector cells to impair access of phenylephrine to sites of

deamination. The possibility that this is a reflection of a general inhibition of the access of sympathomimetic amines to sites of enzymatic degradation is suggested by the finding that tropolone and cocaine inhibited 65% and 35%, respectively, of the inactivation of adrenaline (1×10^{-8}), but the combination produced only 85% inhibition.

Contractions produced by 3 of the agents included in experiments described in this section, tyramine (after MAO inhibition), bretylium and guanethidine, consistently showed a progressive, marked increase in amplitude after oil immersion. These agents are reputed to act by the release of endogenous catecholamines, and the present results appear to confirm this opinion. The responses were blocked by phenoxybenzamine, indicating that they were mediated by α adrenergic receptors, and although never completely absent, the contractions of reserpinized strips after oil immersion were much smaller than those of unreserpinized preparations. However, the response to the procedure is itself the most convincing evidence of the mechanism involved. The strips were exposed to the agents in question for long periods, and any contraction produced while in the Krebs solution had reached a stable plateau before oil immersion. Equilibrium between bath and tissue concentrations of drug, or a steady state, must have been established during this period, and it is difficult to conceive of a mechanism by which the pertinent receptors could be presented with a higher concentration of the drug itself as a result of replacing the aqueous medium with oil. However, there would have been a steep diffusion gradient from the tissue to the bathing medium for any material released from endogenous stores; the oil would prevent its escape from the tissue, and even very slow release might lead to effective concentrations in the

tissue.

The extreme sensitivity of the oil immersion technique in detecting small amounts of catecholamine was illustrated by the fact that even when there was no detectable response in Krebs solution, e.g., reserpinized strips exposed to any one of the 3 agonists and many unreserpinized strips exposed to guanethidine, a contraction of considerable magnitude was recorded after oil immersion. This sensitivity appeared to be further increased by inhibition of mechanisms for the inactivation of released mediator, as in strips treated with the combination of iproniazid, tropolone and cocaine. Their slow contraction after oil immersion probably reflects the spontaneous release, or leakage, of mediator in the absence of nerve activity. On the basis of the contraction produced, this led to a tissue concentration of noradrenaline 1×10^{-9} over a period of 30 minutes in oil. In experiments on the release of noradrenaline in aortic strips from reserpinized rabbits by GD-131, the oil immersion technique easily detected a concentration equivalent to 4×10^{-10}, which in a strip with a volume of about 20 mm^3, represents a clear effect of release at a rate of about 16 picograms/hour. The minimum detectable in such a strip probably is about 2 picograms.

In contrast to the other indirectly acting agents studied, guanethidine produced an effect which was not readily reversed by washing the tissue, as indicated by the contractile responses obtained by intermittent reimmersion in oil without re-exposure to guanethidine. A similar persistence of the catecholamine potentiating action of guanethidine in aortic tissue after washout was reported by Maxwell et al. (1962).

The observation that concentrations of tyramine, guanethidine and bretylium which produced only small contractions of aortic strips, did so by the release of endogenous noradrenaline, provides strong support for the belief that this is an important component of their action, and not the result of excessive dosage, as has been recently suggested (Zaimis, 1964). However, these findings are not incompatible with the presence of an additional direct action of tyramine on effector cells (e.g., Nasmyth, 1960).

In the past, evaluation of the contributions of various inactivation mechanisms for sympathomimetics and other smooth muscle stimulants has been based on the potentiation of responses of various organs in vivo or in an aqueous medium in vitro produced by inhibition of the mechanism in question. It is of interest to apply this procedure to some of the present observations on potentiation, and to compare estimates of the importance of various inactivation processes derived from them with the values obtained by the more direct procedure of oil immersion.

Although studies on rates of relaxation after oil immersion clearly demonstrated that MAO was the major endogenous route for the inactivation of phenylephrine, potentiation of responses to this agent by iproniazid was most difficult to demonstrate. Only with an intra-individual comparison to reduce variance, was it possible to show a statistically significant, but minor effect. This difficulty was not due to masking of the potentiation by α adrenergic blockade. Although high concentrations of iproniazid can produce some blockade, it would not be expected to appear under the conditions of these experiments

(Kamijo et al., 1956), and this was confirmed by experiments on Cobe-
frine, run in parallel with those on phenylephrine. Thus, on the
basis of typical in vitro experiments, deamination would have been
incorrectly considered to be unimportant in the inactivation of phenyl-
ephrine.

The contribution of diffusion into the bathing medium appears
to provide an obvious explanation for the disparity between this con-
clusion and the 85 to 90% reduction in the rate of disposition of
phenylephrine after inhibition of MAO shown by oil immersion. This
degree of reduction in inactivating capacity should have resulted in
a contraction equivalent to that produced by a 6 to 10 times higher
concentration of amine. However, studies on the behavior of phenyl-
ephrine contracted strips in Krebs solution (Section V, a) showed that
as the concentration of amine used to produce contractions decreased,
the contribution of diffusion into the bathing medium to the termina-
tion of its action after washout increased. Thus, at the lower concen-
trations of agonist, the rate constant for diffusion out of the tissue
appears to have been greater than that for deamination, and this pro-
bably prevented observations on potentiation from detecting the major
role of MAO in the endogenous inactivation of phenylephrine.

Inhibition of COMT decreased the capacity of aortic strips to
inactivate adrenaline (1×10^{-8}) by about 65%, as determined by the oil
immersion technique, and potentiated responses to it to the equivalent
of those produced by a 3 times higher concentration of the amine. This
is a greater potentiation relative to the degree of inhibition of in-
activation than was observed in the study of the effects of inhibition

of MAO on responses to phenylephrine, and suggests that COMT competes more effectively with diffusion as a mechanism terminating the action of sympathomimetics in aortic strips suspended in an aqueous medium than does MAO. Additional support for this interpretation is found in the results showing that inhibition of MAO in addition to COMT caused a considerable additional delay in the inactivation of adrenaline, but no further potentiation of responses to it. Thus, it appears that the morphological location of an endogenous process for the inactivation of amines, and perhaps its specific relations to various diffusion barriers, may be an important factor determining whether and to what extent its inhibition is reflected in potentiation of responses to the agonist.

As pointed out above, the presence and degree of potentiation of responses to an agonist after inhibition of a potential inactivating mechanism is the criterion currently used by most investigators in the field of adrenergic mechanisms to assess the importance of that process in terminating its action. The results presented and discussed in this section show that the absence of potentiation does not prove a given mechanism to be unimportant, or even that it is not the dominant endogenous mechanism terminating the action of an agonist. Two factors, in particular, appear to contribute to this discrepancy.

1) The presence of alternative mechanisms, which, as in the case of MAO in the inactivation of catecholamines by COMT, can mask both potentiation and the normal contribution of the pathway to amine inactivation.

2) The contribution of diffusion of agonist into the surrounding medium, which can compete with varying degrees of effectiveness with

various endogenous mechanisms. It appears that the importance of diffusion relative to endogenous mechanisms depends on the relative rate constants. Thus, the manner in which a sympathomimetic is presented, its concentration and the anatomical localization of endogenous inactivation mechanisms can determine whether or not observations on potentiation provide a useful criterion of the roles of potential endogenous terminating mechanisms under physiological conditions.

Results will be presented in the next sections of this thesis to show the converse of the above, that clear potentiation of responses to an agonist by a drug capable of inhibiting some mechanism of inactivation can occur entirely independently of the effect on inactivation, and, therefore, that positive evidence of potentiation cannot be considered to be a reliable indication of interference with an important mechanism of endogenous inactivation of sympathomimetic amines.

VI. EFFECTS OF RESERPINE PRETREATMENT ON ADRENERGIC
MECHANISMS

The process by which adrenergic neurones "take up" and store noradrenaline and other sympathomimetic amines is considered to have two components. The first is a nerve membrane transport mechanism which ferries extracellular amine into the cell, and the second, the process of incorporation of intraneuronal amine into storage granules, or vesicles. Cocaine is believed to block transport at the nerve membrane and reserpine to inhibit incorporation into storage granules (Furchgott et al., 1963; Kopin, 1964; Malmfors, 1965; Carlsson and Waldeck, 1965; Dahlström et al., 1965). The most prominent effect of reserpine is depletion of tissue stores of catecholamine, but the extent to which this is due to inhibition of incorporation into storage units is still unclear. Reserpinization has been reported to alter catecholamine metabolism in such a way that amine which would normally be stored in granules is, instead, deaminated intraneuronally (Kopin and Gordon, 1962, 1963; Stjärne, 1964; Kopin, 1964), and to potentiate responses of various sympathetically innervated tissues to catecholamines and certain other sympathomimetics. (See review by Trendelenburg, 1963.)

The experiments to be reported in this section were undertaken to determine: (1) if the supersensitivity of reserpinized aortic strips to sympathomimetic amines is due to a decreased rate of inactivation of the agonist, with a consequent increase in the concentration at the receptors, and (2) if the contributions of the cocaine sensitive mechanism and of deamination by MAO to the inactivation of sympathomimetic amines are altered by reserpinization.

A. RESULTS

1. Reserpine Doses and Treatment Schedules:

Reserpine was prepared as described in Methods. Rabbits were injected intramuscularly either with 2 doses of 0.5 mg/kg, 48 and 24 hours before death, or with one dose of 5.0 mg/kg, 18 to 24 hours before death. The completeness of endogenous catecholamine depletion in aortic strips from reserpinized animals was not checked routinely. However, the doses of reserpine used were well above those reported to produce essentially complete depletion, and a comparison in our laboratory, by the trihydroxyindole fluorimetric method, showed a catecholamine level of 1.14 µg/g (noradrenaline 1.06 µg/g; adrenaline 0.08 µg/g) in the aorta of a control animal and no catecholamine detectable by this method in the aorta of a rabbit treated with reserpine (0.5 mg/kg, daily for 2 days).

2. Responses to Sympathomimetic Amines:

Aortic strips from reserpinized rabbits responded to standard concentrations of a number of sympathomimetic amines with greater contractions than did control strips from untreated animals. For example, 6 control strips and 8 from reserpinized animals responded to Cobefrine (1×10^{-8}) with mean contraction amplitudes of 2.2 and 10.1 mm, respectively. Responses to noradrenaline and phenylephrine were similarly potentiated (Table XII). No differences between the responses of aortic strips from animals on the two reserpine dosage schedules was detected in these studies, and the results obtained with all reserpinized strips have been combined in the table.

TABLE XII

COMPARISON OF RESPONSES TO SEVERAL SYMPATHOMIMETICS ON

UNRESERPINIZED AND RESERPINIZED AORTIC STRIPS

Agonist	Contraction Amplitude (mm)	
	Unreserpinized	Reserpinized
Cobefrine (1×10^{-8})	2.2 ± 0.4 (6, 2)	10.1 ± 1.1 (8, 3) $P < 0.001$
Phenylephrine (3×10^{-9})	3.3 ± 1.1 (16, 5)	18.1 ± 1.4 (14,5) $P < 0.001$
(1×10^{-8})	12.5 ± 0.7 (24, 7)	28.0 ± 1.2 (4, 3) $P < 0.001$
(3×10^{-8})	27.1 ± 1.4 (16, 11)	35.6 ± 2.9 (10,6) $P < 0.01$
Noradrenaline (1×10^{-8})	28.3 ± 2.8 (10, 10)	38.9 ± 3.5 (8, 8) $P < 0.05$

In parentheses are indicated number of strips and number of animals
from which they were obtained, respectively.

3. _Relaxation of Phenylephrine Contracted Strips_:

Sixteen strips from 4 reserpinized rabbits were studied by the same procedures described above for unreserpinized preparations, and the rates of relaxation in the 2 groups compared (Fig. 34). The mean contraction height of 4 control reserpinized strips before oil immersion was 33.5 mm. Reserpinized and unreserpinized strips relaxed in oil at comparable rates, the process being 36.8 and 38.8% complete, respectively, in 5 minutes. Reserpinization had no apparent effect on the ability of either cocaine or iproniazid to delay the relaxation of strips contracted by phenylephrine. Cocaine (1×10^{-5}) increased the intervals required for half relaxation to 1.94 and 1.76 times those of controls in reserpinized and unreserpinized strips, respectively. Similarly, iproniazid pretreatment increased the periods required for reserpinized and unreserpinized strips to relax 20% (50% relaxation was not achieved during the 30 minute period studied) to 10.9 and 9.3 times those of their controls, respectively.

Iproniazid pretreated strips from reserpinized animals, contracted with phenylephrine (3×10^{-8}) and treated with cocaine (1×10^{-5}) behaved in oil as did unreserpinized strips treated in the same way. No significant relaxation was evident after 30 minutes of oil immersion in either group.

A comparison of complete phenylephrine dose-response curves determined on reserpinized and unreserpinized aortic strips (Fig. 1) showed the maximal response of the former to be greater. Because of this difference and the general potentiation of responses to phenyl-ephrine by reserpinization, any point on the relaxation curve repre-
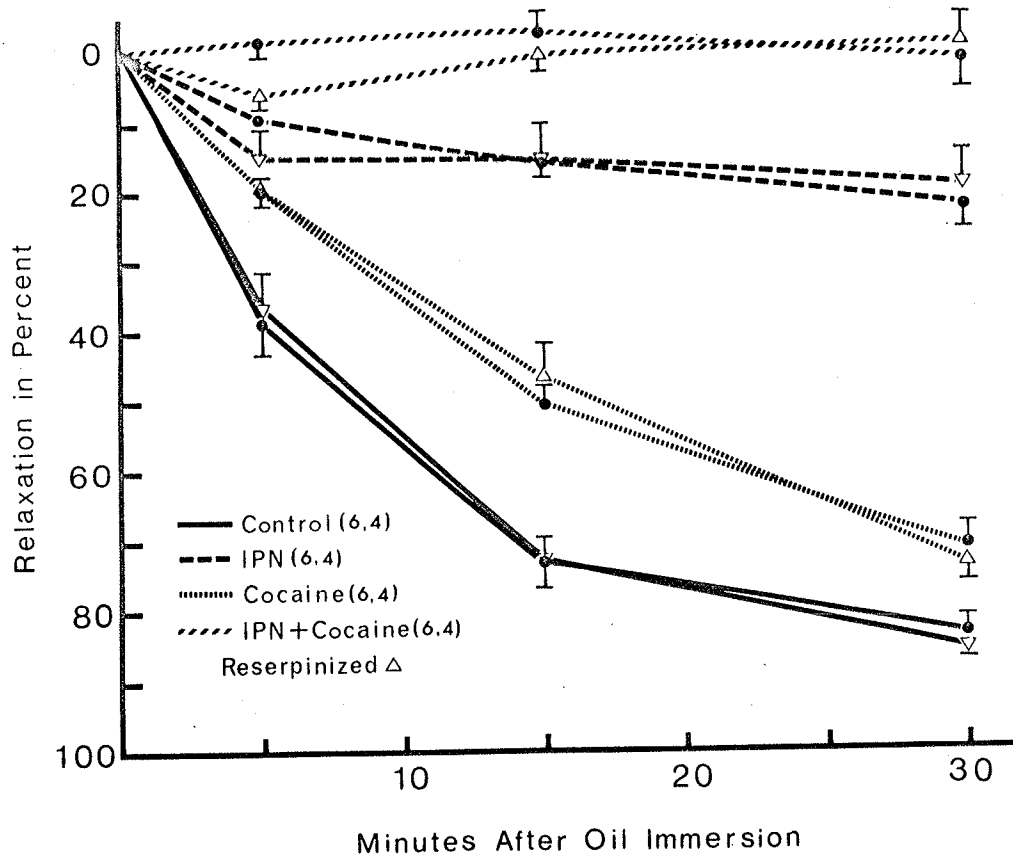
Fig. 34. Effects of Reserpine, Iproniazid (IPN) and Cocaine on Relaxation of Phenylephrine Contracted Aortic Strips after Oil Immersion.

All strips were contracted by phenylephrine (3×10^{-8}); cocaine concentration was 1×10^{-5}. Figures in parentheses indicate number of unreserpinized and reserpinized preparations, respectively, represented by the curves. Bars indicate standard errors of means.

sents different residual amine concentrations in the unreserpinized

and reserpinized strips. Thus, the effective concentrations of the

sympathomimetic, determined from the dose-response curves, at 10 and

30% relaxation of unreserpinized strips are equivalent to those

present at 8.3 and 26.2% relaxation in reserpinized strips. This

disparity increased with further relaxation, 50 and 39.3% relaxation

representing equivalent concentrations of phenylephrine in unreserpinized

and reserpinized strips, respectively. However, the untreated strips

relaxed 50% in 8.2 $\pm$ 1.2 and the reserpinized strips 39.3% in 5.35 $\pm$ 0.9

minutes, a difference in time which is not statistically significant

($P < 0.2 > 0.1$), and it appears that comparisons of the various curves

of relaxation in oil are not seriously compromised by this disparity.

4.   Effect of Iproniazid on Contraction Amplitude in Response to

Phenylephrine and Cobefrine:

Iproniazid pretreatment did not significantly alter the

magnitude of responses of reserpinized strips to phenylephrine

(3×10^{-8}). The peak contractions of 10 control and 8 iproniazid

pretreated strips were 35.6 $\pm$ 2.9 and 38.6 $\pm$ 4.0 mm, respectively.

Possible effects of inactivation of phenylephrine by MAO on the con-

tractions produced by this sympathomimetic in strips from reserpinized

rabbits were further checked by an intraindividual comparison of

responses to phenylephrine (3×10^{-9}) and Cobefrine (1×10^{-8}) before

and after exposure to iproniazid, as described previously for un-

reserpinized strips (Section V, A). The treatment with iproniazid had

no significant effect on the amplitude of contractions produced by

either sympathomimetic (Table XIII).

TABLE XIII

EFFECT OF IPRONIAZID (IPN) ON CONTRACTION OF RESERPINIZED AORTIC STRIPS

BY PHENYLEPHRINE AND COBEFRINE

Agonist	First Contraction (mm)	Interval Treatment	Second Contraction (mm)	Change in Response
Cobefrine 1 x 10^{-8}	9.8 (6 - 12)	--	7.0 (4 - 10)	- 28.5 $\pm$ 14% (4)
	10.5 (5 - 13)	IPN	7.5 (4 - 11)	- 27.3 $\pm$ 9% (4)
				N.S.
Phenyl-ephrine 3 x 10^{-9}	17.9 (12 - 27)	--	13.7 (3 - 23)	- 27.0 $\pm$ 15% (7)
	18.4 (13 - 26)	IPN	16.7 (3 - 24)	- 11.0 $\pm$ 18% (7)
				N.S.

Figures in parenthesis on the extreme right indicate number of strips. Figures appearing to the right of contraction amplitudes give the range of values.

5. Effect of Cocaine on Contraction Amplitude in Response to

Phenylephrine:

Cocaine (1×10^{-5}) added to the chambers after contractions

produced by phenylephrine (3×10^{-8}) had reached a plateau value

increased the contractions of 6 strips from reserpinized animals a

mean of 4.2 mm (13.3%). This potentiated response is equivalent to

the contraction produced by a concentration of 6.4×10^{-8} phenyl-

ephrine. Five reserpinized strips pretreated with iproniazid responded

to cocaine with an increment of 3.8 mm (13.4%). Thus, neither

reserpinization per se nor inhibition of MAO in strips from reserpine

pretreated animals altered the action of cocaine which results in

potentiation of responses to phenylephrine.

6. Relaxation of Noradrenaline Contracted Strips:

The procedures used to assess tissue mechanisms for the

inactivation of noradrenaline in aortic strips from reserpinized

rabbits were identical to those used in studies on unreserpinized

strips (Section V, B). The results of experiments on 60 strips from

9 animals are shown in Table XIV. As in the case of phenylephrine,

each point on the relaxation curves of noradrenaline contracted strips

corresponds to a different effective amine concentration in reserpinized

and unreserpinized tissues. For example, the residual concentrations

of noradrenaline, determined from dose-response curves (Fig. 1), at 10

and 30% relaxation of unreserpinized strips were equivalent to those

present at 9.0 and 25.8% relaxation of reserpinized strips. As for

phenylephrine, this disparity increased with progressively greater

relaxation, 50 and 44% relaxation representing equivalent concentrations

Statistical Analysis of Results Summarized in Table XIV.

	Min.
Control vs. cocaine (1×10^{-5})	5 – P < 0.1
Control vs. iproniazid	N.S.
Control vs. tropolone	5 – P < 0.001 15 – P < 0.01 30 – P < 0.05
Iproniazid vs. iproniazid + cocaine (1×10^{-5})	N.S.
Iproniazid + tropolone vs. tropolone	5 – P < 0.001 15 – P < 0.001 30 – P < 0.001
Tropolone + cocaine (1×10^{-5}) vs. tropolone	5 – P < 0.02 15 – P < 0.01 30 – P < 0.1
Iproniazid + tropolone + cocaine (1×10^{-5}) vs. iproniazid + tropolone	N.S.
Iproniazid + tropolone + cocaine (1×10^{-4}) vs. iproniazid + tropolone	5 – P < 0.1 30 – P < 0.05
Cocaine (1×10^{-5}) vs. cocaine (1×10^{-4})	5 – P < 0.02

TABLE XIV

RELAXATION OF RESERPINIZED NORADRENALINE CONTRACTED AORTIC STRIPS AFTER OIL IMMERSION

Treatment Condition	5 Min.	15 Min.	30 Min.
Control (8)	54.6 ± 3.2	81.6 ± 1.6	89.4 ± 0.5
Cocaine (1×10^{-5}) (7)	44.0 ± 3.9	76.3 ± 2.8	88.9 ± 1.8
Cocaine (1×10^{-4}) (8)	28.8 ± 3.6	69.0 ± 3.4	86.8 ± 1.2
Iproniazid (6)	49.2 ± 3.7	78.8 ± 1.6	90.3 ± 1.0
Tropolone (7)	32.0 ± 3.9	67.7 ± 3.5	85.6 ± 1.5
Iproniazid + Cocaine (1×10^{-5}) (5)	47.0 ± 2.6	76.8 ± 2.2	90.6 ± 0.97
Iproniazid + Tropolone (6)	11.0 ± 1.8	23.3 ± 5.2	42.3 ± 6.3
Tropolone + Cocaine (1×10^{-5}) (5)	19.0 ± 1.4	52.8 ± 2.5	80.2 ± 2.5
Iproniazid + Tropolone + Cocaine (1×10^{-5}) (6)	8.0 ± 1.8	14.0 ± 4.2	27.0 ± 6.4
Iproniazid + Tropolone + Cocaine (1×10^{-4}) (2)	3.5 ± 1.5	6.0 ± 1.0	12.0 ± 2.0

Values shown are mean percent relaxations at the indicated times after oil immersion.
Number of complete experiments are indicated in parentheses.

of active amine. Unreserpinized strips relaxed 50% in 5.29 $\pm$ 0.5 minutes and reserpinized strips 44.0% in 4.0 $\pm$ 0.5 minutes. This difference in time was not significant at the 5% level of probability ($P < 0.1 > 0.05$), but may reflect a real difference. Reserpinization did not clearly or markedly increase the ability of aortic tissue to inactivate noradrenaline, but because of this disparity, comparisons of the effects of various treatments on the relaxation rates of reserpinized and unreserpinized strips are presented in terms of change in "equivalent concentration", relative to the appropriate controls (Table XV).

Iproniazid pretreated and control reserpinized strips relaxed at comparable rates, the process being 49.2 and 54.6% complete after 5 and 78.8 and 81.6% after 15 minutes of oil immersion in the two groups. Reserpinized strips treated with cocaine (1×10^{-5}) relaxed 44.0% in 5 and 76.3% in 15 minutes. The difference from the behavior of control reserpinized strips was greatest 5 minutes after oil immersion, but this was significant only at the 10% level of probability. Cocaine (1×10^{-5}) increased the period required for 50% relaxation of reserpinized and unreserpinized strips to 1.36 and 1.49 times those of their controls, respectively.

The COMT inhibitor, tropolone, slowed the relaxation of reserpinized more than that of unreserpinized strips, the periods required for 50% relaxation being increased to 2.09 and 1.33 times those of their controls, respectively. Reserpinized strips treated with both iproniazid and tropolone relaxed much more slowly than did similarly treated unreserpinized strips. The combined treatment

TABLE XV

RELAXATION OF RESERPINIZED AND UNRESERPINIZED AORTIC STRIPS

CONTRACTED BY NORADRENALINE (1×10^{-8})

Treatment	UNRESERPINIZED			RESERPINIZED		
	No. Exps.	Time to Relax 50% (min.)	Shift from Control Time	No. Exps.	Time to Relax 50% (min.)	Shift from Control Time
Control	10	5.29 ± 0.5 1.75 ± 0.1*		8	4.74 ± 0.5 1.68 ± 0.2*	
Iproniazid	10	5.14 ± 0.3		6	5.37 ± 0.7	1.13
Tropolone	6	7.03 ± 0.7	1.33	7	9.89 ± 1.2	2.09
Cocaine	9	7.86 ± 1.3	1.49	7	6.43 ± 0.7	1.36
Cocaine (1×10^{-4})	2	8.22 ± 1.1	1.55	8	8.73 ± 1.1	1.84
Iproniazid + Tropolone	7	17.0 ± 3.1	3.21	5	32.42 ± 3.8	6.84
Iproniazid + Cocaine	8	8.13 ± 1.2	1.54	5	6.00 ± 0.7	1.27
Tropolone + Cocaine	5	13.88 ± 0.7	2.62	5	13.98 ± 1.0	2.95
Iproniazid + Tropolone + Cocaine	9	15.15 ± 2.3*	8.66	6	26.2 ± 5.0*	15.6
Iproniazid + Tropolone + Cocaine (1×10^{-4})	6	34.28 ± 10.2*	19.58	2	37.85 ± 1.5*	22.5

Cocaine concentration was 1×10^{-5} except where otherwise indicated.

* Indicates time to relax 20% and corresponding shift from control values at 20% relaxed.

increased the interval required for reserpinized strips to relax 50%

to 6.84 times that of controls, whereas the time for 50% relaxation of

unreserpinized strips was increased to only 3.21 times the control value.

Reserpinized strips treated with iproniazid, tropolone and

cocaine (1×10^{-5}) relaxed in oil at a rate significantly slower than

that of strips treated with tropolone plus cocaine, but not signifi-

cantly different from that of strips treated with iproniazid plus

tropolone. However, strips treated with the two enzyme inhibitors

plus cocaine (1×10^{-4}) did relax at a significantly slower rate than

did strips treated with the enzyme inhibitors alone.

7. Effects of Tropolone, Iproniazid and Cocaine on Contraction

Amplitude in Response to Noradrenaline:

The effects of iproniazid and tropolone on the magnitude of

responses to noradrenaline were assessed on the basis of all peak

contractions obtained after treatment with these inhibitors, including

those of strips which subsequently were exposed to cocaine (Table XVI).

As in unreserpinized strips, neither iproniazid, tropolone nor the two

together significantly altered the amplitude of contractions produced

by noradrenaline in aortic strips from reserpinized animals. Strips

exposed to both iproniazid and tropolone and their controls responded

to noradrenaline (1×10^{-8}) with almost identical peak contractions,

38.3 ± 4.3 and 37.3 ± 2.5 mm, respectively.

Potentiation of responses to noradrenaline by cocaine was

determined on reserpinized strips in the same way as on unreserpinized

preparations (Section V, B). The results are shown in Table XVI.

Contractions of 7 control reserpinized strips in response to nor-

TABLE XVI

EFFECTS OF IPRONIAZID, TROPOLONE AND COCAINE ON NORADRENALINE (1×10^{-8})

CONTRACTION AMPLITUDE OF RESERPINIZED AORTIC STRIPS

Treatment	Noradrenaline Contraction (mm)	Cocaine Increment 1×10^{-5}* mm	%	1×10^{-4}* mm	%	Equiv. Conc. Noradrenaline 1×10^{-5}*	1×10^{-4}*
Control	37.3 ± 2.5 (15)	7.0 (7)	19.1 ± 2.6	11.4 (8)	27.8 ± 2.5	2.3×10^{-8}	3.6×10^{-8}
Iproniazid	37.8 ± 2.2 (11)	7.6 (5)	20.8 ± 2.3	--	---	---	---
Tropolone	34.5 ± 3.7 (11)	5.5 (4)	18.3 ± 4.1	--	---	---	---
Iproniazid + tropolone	38.3 ± 4.3 (12)	4.7 (6)	12.2 ± 2.2	7.0 (2)	16.5 ± 2.5	---	---

All heights refer to peak heights. Equivalent concentrations of noradrenaline are shown for control cocainized strips and for all groups which differ significantly. Contraction amplitudes of iproniazid, tropolone and iproniazid plus tropolone treated strips not significantly different from the control.

Statistical Analysis (cocaine increments):

Cocaine (1×10^{-5}) vs. cocaine (1×10^{-4})	$P < 0.05$
Cocaine (1×10^{-5}) vs. iproniazid + tropolone + cocaine (1×10^{-5})	$P < 0.1 > 0.05$
Cocaine (1×10^{-4}) vs. iproniazid + tropolone + cocaine (1×10^{-4})	$P < 0.1 > 0.05$

* Concentration of Cocaine

adrenaline (1×10^{-8}) were increased 7.0 mm ($19.1 \pm 2.6\%$) by cocaine
(1×10^{-5}), compared to a 4.6 mm ($18.3 \pm 1.6\%$) increase in the
responses of comparable unreserpinized strips. The difference in per-
cent augmentation is clearly not statistically significant, and in both
groups, the potentiated responses were approximately equivalent to
those which would be produced by doubling the concentrations of nor-
adrenaline. As was found in the study of unreserpinized aortic strips,
neither iproniazid nor tropolone pretreatment altered the potentiation
due to cocaine, and treatment with both enzyme inhibitors tended to
decrease the augmentation due to cocaine. The contractions of 6
reserpinized strips pretreated with both enzyme inhibitors were
increased $12.2 \pm 2.2\%$ and $16.5 \pm 2.5\%$ by cocaine in concentrations of
1×10^{-5} and 1×10^{-4}, respectively. These effects differed from those
on reserpinized strips not treated with the enzyme inhibitors only at
the 10% level of probability, a relationship similar to that found in
unreserpinized strips.

B. DISCUSSION

It is now widely accepted that reserpine inhibits the
accumulation of catecholamines in adrenergic nerves by blocking
incorporation of the amines into intraneuronal granules (Kopin and
Gordon, 1963; Furchgott et al., 1963; Kopin, 1964; Carlsson and Waldeck,
1965; Malmfors, 1965). Reserpinization also potentiates responses of
a variety of effectors to sympathomimetic amines, and this effect has
been assumed by many workers to result from decreased amine inactivation

by the nerve uptake mechanism, which allows a higher proportion of the available agonist to reach tissue receptors (MacMillan, 1959; Brodie and Beaven, 1963; Axelrod, 1965; Hertting, 1965). However, this view has never been satisfactorily reconciled with the fact that cocaine still effectively potentiates responses of reserpinized preparations to sympathomimetic amines.

The effects of various treatments on the rates of inactivation of noradrenaline (1 X 10^{-8}) in reserpinized and unreserpinized aortic strips are compared in Table XV. These results are compatible with the hypothesis that uptake and storage in adrenergic neurones plays a less important role in the inactivation of noradrenaline in reserpinized than in normal tissues. Cocaine, alone and in various combinations with other agents, slowed the relaxation in oil of reserpinized aortic strips less than that of unreserpinized preparations. However, a definite contribution of the cocaine sensitive mechanism to the inactivation of noradrenaline and certain other sympathomimetic amines remained after reserpinization. This was indicated by the decreased rates of relaxation of reserpinized aortic strips in oil caused by exposure to cocaine, either alone or after treatment with tropolone or tropolone plus iproniazid. However, both alone and in combination with both enzyme inhibitors, cocaine produced a statistically significant slowing only in the relatively high concentration of 1 X 10^{-4}, whereas in unreserpinized strips its effect on rate of relaxation was obvious at 1 X 10^{-5}.

These observations suggest that reserpine and cocaine affect overlapping components of some process of amine inactivation. These

could be the two steps in intraneuronal storage, nerve membrane trans-
port and intraneuronal incorporation into granules, on which cocaine
and reserpine, respectively, are now generally assumed to act. However,
the results also indicate that a part of the effect of cocaine on amine
inactivation in reserpinized preparations is operative even after
inactivation of both MAO and COMT. It is possible that at least a part
of this independent action of cocaine is on processes leading to extra-
neuronal binding and storage of amine.

It currently is generally assumed that inhibition of storage
in intraneuronal granules by reserpine diverts catecholamine to
metabolic inactivation via intraneuronal MAO. However, most studies
have provided no direct evidence regarding the site of inactivation,
and intraneuronal deamination is assumed on the basis of early
experiments showing that much of the endogenous catecholamine released
by reserpine is deaminated and that the overt responses of various
effectors are much less than would be expected if most of the mediator
released left the nerves in active form (Chessin, et al., 1957; Kopin
and Gordon, 1962, 1963; Stjärne, 1964). The present experiments
provided no evidence that reserpinization increased the role of MAO in
the inactivation of noradrenaline. The time for half relaxation of
reserpinized strips contracted by noradrenaline (1×10^{-8}) was not
increased significantly by pretreatment with iproniazid, to only 1.13
times that of controls. When added to treatment with both tropolone
and cocaine (1×10^{-5}), iproniazid did significantly inhibit the
relaxation in oil of both reserpinized and unreserpinized strips
contracted by noradrenaline. However, this effect was not increased by

reserpinization, and it probably reflects predominantly the inhibition of extraneuronal MAO because the strips were treated with a dose of cocaine sufficient to reduce markedly the transport of noradrenaline into nerves.

In contrast to the effect generally assumed, the present results indicate that inhibition of storage by reserpinization diverts amine to metabolic inactivation by catechol-O-methyl transferase (COMT) rather than by MAO. The relatively greater role of COMT as a mechanism terminating the action of noradrenaline in reserpinized than in un-reserpinized aortic strips was seen in the effects of several treatments. Tropolone alone caused a greater increase in the time required for half relaxation of the former, and when added in combination with iproniazid, it very markedly slowed the relaxation of strips contracted by nor-adrenaline. In view of the predominantly extraneuronal location of COMT, it appears that this finding might be accounted for by one or more of the following: 1) Some of the storage or binding sites which are inactivated by reserpine and access to which is prevented by cocaine are extraneuronal. 2) There is some intraneuronal COMT, which is only apparent after blockade of uptake into storage granules. 3) Amine which is not stored intraneuronally diffuses out of the nerves to sites of COMT activity without being deaminated by MAO. 4) Reserpinization leads to an actual increase in COMT activity.

The results presented in this section are not in agreement with the concept that the sensitization to sympathomimetic amines produced by reserpine results from decreased inactivation, which allows more amine to reach the tissue receptors. No decreased rate of inacti-

vation attributable to reserpine was found in any experiment. The
results suggest, in fact, that both phenylephrine and noradrenaline
may be somewhat more rapidly inactivated in strips from reserpinized
than in those from unreserpinized animals. The increases in rates of
relaxation in oil measured in the present experiments were not
statistically significant. However, the direction of the differences
observed serves to emphasize the absence of the marked decrease in ꞌ
total rates of inactivation required to explain sensitization by
reserpine on this basis.

Although it is believed to act at the nerve cell membrane
rather than at storage sites within the nerve, cocaine, like reserpine,
causes both a decrease in the inactivation of sympathomimetic amines by
uptake and storage in nerves and potentiation of responses to the
amines. It is generally believed that the latter is a consequence of
the former effect. However, the present results appear to be incom-
patible with this interpretation. (See also Section VII, A.) Cocaine
was found to potentiate responses to noradrenaline as effectively in
reserpinized as in unreserpinized aortic strips (Table XVI), and pre-
treatment with iproniazid, tropolone, or a combination of both enzyme
inhibitors did not clearly alter its efficacy. The observations on
reserpinized aortic strips treated with both enzyme inhibitors are of
particular importance in assessing the mechanism of action of cocaine.
Contractions produced in such strips by noradrenaline were clearly
potentiated by cocaine (1 X 10^{-5}), which had no significant effect on
the rate of inactivation of noradrenaline, as measured by relaxation
in oil. These findings strongly suggest that the decreased rate of

inactivation of amines and the potentiation of responses to them by cocaine are reflections of two independent actions of the drug.

VII. RESULTS OF STUDIES ON MECHANISMS OF POTENTIATION

A. POTENTIATION OF RESPONSES TO SYMPATHOMIMETIC AMINES BY COCAINE

Experiments reported in Section V, A showed cocaine to potentiate the responses of aortic strips from reserpinized and unreserpinized rabbits to phenylephrine about equally, an effect equivalent to doubling the concentration of agonist. This is compatible with the currently dominant interpretations of adrenergic mechanisms, which gives a key role in the regulation of responses to the uptake and storage of amines by adrenergic nerves. Cocaine is believed to potentiate responses to sympathomimetics by blocking inward transport of amine at the nerve cell membrane, with a resultant diversion from intraneuronal storage or destruction to the vicinity of the adrenergic receptors, and reserpine to block the deposition of amine from nerve cell cytoplasm in storage granules, but not to alter transport into the nerve (Furchgott et al., 1963; Kopin, 1964; Trendelenburg, 1965; Dahlström et al., 1965; Carlsson and Waldeck, 1965). Thus, the "unitary" theory of adrenergic mechanisms implies that the amount of amine entering adrenergic nerves is unaltered by reserpine, but that after reserpinization the intraneuronal amine is inactivated enzymatically rather than by storage. On this basis it would be expected that after inhibition of monoamine oxidase (MAO), the concentration of a sympathomimetic such as phenylephrine, which does not have alternative pathways of metabolic inactivation, in the nerve cell cytoplasm would rapidly come to equilibrium with that in the extracellular fluid, and inhibition of nerve cell membrane transport would no longer potentiate the response. However, as described above, inhibition of MAO in reserpinized strips did not reduce the augmentation of responses to phenylephrine by

cocaine. If the sensitizing action of cocaine is due to inhibition of

uptake of amine by nerves, the only explanation for this observation

is that, even in the absence of both enzymatic degradation and storage

in granules, the nerve cell cytoplasm can accommodate a major part of

the amine entering the tissue. This possibility could not be evaluated

on the basis of the observations on mechanisms of inactivation of

phenylephrine reported in Section V, A, and was investigated further.

1. Effect of Cocaine on Responses to Phenylephrine:

Contractions were produced by phenylephrine in iproniazid

pretreated aortic strips from reserpinized rabbits. As in the previous

studies, the two schedules of reserpinization used (0.5 mg/kg, intra-

muscularly, 48 and 24 hours before death, and a single dose of 5.0 mg/kg,

intramuscularly, 16 to 24 hours before death) gave comparable results.

Cocaine (1×10^{-5}) was added to the bath after responses to phenyl-

ephrine (1×10^{-8}) had reached plateau values. The chambers were then

washed out, the strips allowed to return to basal tone, exposure to

phenylephrine repeated for a longer period, and cocaine again added to

the muscle chambers. Records from a typical experiment are shown in

figure 35a. It was expected that neuronal cytoplasmic accumulation of

phenylephrine occurring in the absence of enzymatic degradation or

granule storage would be limited in extent, and that outward diffusion

would soon diminish net uptake and decrease the importance of membrane

transport as a mechanism of inactivation. However, cocaine augmented

the responses just as well after 60 as after 10 minutes of exposure to

phenylephrine. This observation strongly suggests that some mechanism

other than inhibition of nerve cell membrane transport is involved in

a.

b.

c.
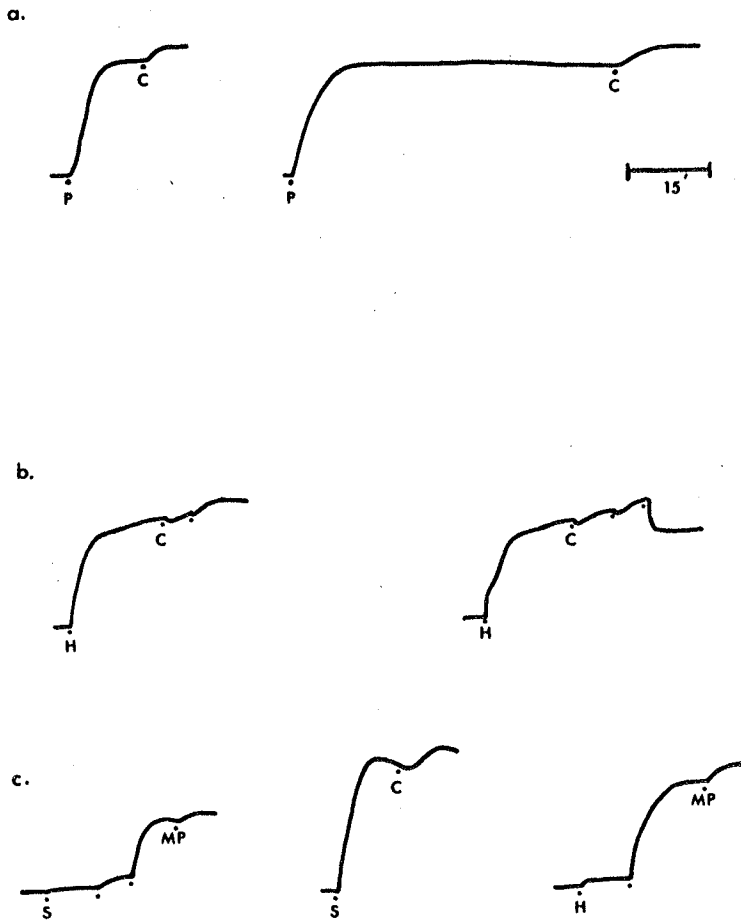
Fig. 35. Potentiation of Responses to Phenylephrine, Histamine and 5-Hydroxytryptamine by Cocaine and Methylphenidate.

a. Responses of a strip from a reserpinized aorta to cocaine (C) (1×10^{-5}); left, after an exposure to phenylephrine (P) (1×10^{-8}) of about 10 min.; right, after a second exposure of about 60 min. b. Left, response of a histamine (H) (1×10^{-6}) contracted strip to cumulative concentrations of cocaine (1×10^{-5}, 1×10^{-4}); right, response of the same strip to a second exposure to cocaine, to a final concentration of 5×10^{-4}. c. Left, response of a strip to cumulative concentrations of 5-hydroxytrypt-amine (S) (1 and 3×10^{-8}, 1×10^{-7}), followed by methylphenidate (MP) (1×10^{-5}); center, response of a 5-hydroxytryptamine (1×10^{-7}) contracted strip to cocaine (1×10^{-4}); right, response of a strip to cumulative concentrations of histamine (3×10^{-7}, 1×10^{-6}), followed by methylphenidate (1×10^{-5}). All aortic strips were from reserpine pretreated rabbits.

the potentiation of responses to phenylephrine by cocaine.

2. <u>Correlation between Potentiation of Responses to Sympathomimetics</u>

   <u>and Impairment of Amine Inactivation by Cocaine:</u>

   Cocaine (1×10^{-5}) potentiated responses of aortic strips to

phenylephrine, adrenaline and noradrenaline all about 100%, and cocaine

(1×10^{-4}) potentiated responses to noradrenaline about 200%, in terms

of equivalent agonist concentrations. However, in all cases, the

impairment of amine inactivation (slowed relaxation) produced by cocaine

appeared to be less than that required to explain the observed potentia-

tion in terms of the unitary hypothesis, even when diffusion into the

muscle chamber, an alternative mechanism of inactivation, was eliminated

by oil immersion. These results are summarized in Table XVII, and

provide further evidence of the inadequacy of decreased inactivation

(decreased transport into nerve cells) as an explanation for the

potentiation of responses to sympathomimetics by cocaine.

3. <u>Effects of Cocaine on Responses to 5-Hydroxytryptamine and</u>

   <u>Histamine:</u>

   Indications that the augmentation of responses of aortic strips

to sympathomimetic amines by cocaine cannot be adequately explained by

blockade of neuronal uptake of amine were also obtained in experiments

with other agonists. Cocaine was found to potentiate responses of

some aortic strips from both unreserpinized and reserpinized animals to

both histamine and 5-hydroxytryptamine (5-HT), as shown in figure 35, b

and c. Cocaine (1×10^{-5}) potentiated responses to histamine in 4/5

reserpinized and 0/3 unreserpinized strips, and a concentration of

1×10^{-4} was effective in 6/6 reserpinized and 1/1 unreserpinized

TABLE XVII

POTENTIATION AND IMPAIRMENT OF INACTIVATION OF SYMPATHOMIMETIC AMINES BY COCAINE

	Noradrenaline (1×10^{-8})				Adrenaline (1×10^{-8})	Phenylephrine (3×10^{-8})	
	Unreserpinized		Reserpinized		Unreserpinized	Unreserpinized	Reserpinized
Cocaine Conc.	1×10^{-5}	1×10^{-4}	1×10^{-5}	1×10^{-4}	1×10^{-5}	1×10^{-5}	1×10^{-5}
Increment (mm)	4.6 (10)	6.8 (4)	7.0 (7)	11.4 (8)	5.0 (5)	3.1 (10)	4.2 (6)
% of Control	18.3 $\pm$ 1.6	25.3 $\pm$ 5.2	19.1 $\pm$ 2.6	27.8 $\pm$ 2.5	21.1 $\pm$ 3.2	11.7	13.3
Equiv. Agonist (Conc.)	2.2×10^{-8}	3.0×10^{-8}	2.3×10^{-8}	3.6×10^{-8}	2.1×10^{-8}	6×10^{-8}	6.4×10^{-8}
50% Relaxation (min.)	7.86+1.3* (9)	8.22+ 1.1* (2)	6.43+0.7* (7)	8.73+1.1* (8)	6.58 $\pm$ 1.4* (5)	14.5 (6)	17.0 (4)
50% Relaxation Time (X control)	1.49	1.55	1.36	1.84	1.57	1.76	1.94
Inhib. of Inactivation Calc. from Potentiation(%)	50 - 55	65 - 70	55 - 60	70 - 75	50 - 55	50	50 - 55
Inhib. of Inactvation Calc. from Relaxation (%)	30 - 35	35	25 - 30	45 - 50	35 - 40	40 - 45	45 - 50

* Time to relax 50% determined directly for each strip. Data from experiments described in sections V and VI.

strips. Methylphenidate (1×10^{-5}), a compound believed to have the same mechanism of action as cocaine (Furchgott, 1960b; Maxwell et al., 1962), potentiated the response to histamine in 1/2 reserpinized and 0/2 unreserpinized strips.

Responses to 5-HT were potentiated less consistently by cocaine than were those to histamine. Cocaine (1×10^{-5}) clearly potentiated the response to 5-HT in 2/6 reserpinized and 0/8 unreserpinized strips, and a concentration of 1×10^{-4} had a similar effect in 1/3 strips of each type. Methylphenidate (1×10^{-5}) potentiated the response to 5-HT in 1/2 reserpinized and 0/2 unreserpinized strips. Neither methylphenidate nor cocaine affected the basal tone of the aortic strips, in agreement with the findings of Maxwell et al. (1962) and Furchgott et al. (1963).

4. Effect of Methylphenidate on the Relaxation of Histamine Contracted Strips:

Four strips (2 from reserpinized rabbits) were contracted twice with histamine (1×10^{-6}), given either cumulatively or in a single concentration, and their relaxations in oil compared without and then with the addition of methylphenidate (1×10^{-5}), 10 times the concentration used to potentiate responses to phenylephrine. The methylphenidate was added cumulatively or as a single concentration after the histamine contractions had reached a plateau value. All strips were exposed to the highest concentration of methylphenidate (1×10^{-5}) for about 10 minutes before oil immersion.

Even with same strip comparisons to reduce variance and with a high concentration of methylphenidate, no slowing of the relaxation

of histamine contracted strips in oil was detected (Fig. 36). Control

strips relaxed 35.1% in 2, 60.6% in 5 and 85.1% in 15 minutes and the

same strips in the presence of methylphenidate relaxed 30.5% in 2, 56.6%

in 5 and 88.0% in 15 minutes.

5.   Effects of Cocaine on Methoxamine Contracted Aortic Strips:

The results so far presented have provided nothing to support

the hypothesis that potentiation of responses to various amines and

impairment of their inactivation by cocaine are causally related.

Methoxamine was chosen for an additional test of this dissociation

because it is not a substrate for either MAO or COMT, and no alternate

enzymatic pathways of importance for its inactivation in vascular smooth

muscle are known or expected. The only known endogenous mechanism

which might contribute to the inactivation of methoxamine appears to be

transport to storage sites, which is believed to be inhibited by

cocaine.

Aortic strips from reserpinized rabbits were contracted by

methoxamine (5×10^{-8} or 1×10^{-7}) and their relaxation after oil

immersion recorded (Fig. 37). Most responses to both 5×10^{-8} and

1×10^{-7} methoxamine developed slowly. For example, 5 strips cut from

the same aorta and exposed to methoxamine (5×10^{-8}) required a mean

of 22.7 minutes, and 3 exposed to methoxamine (1×10^{-7}) a mean of

24.0 minutes to reach mean plateau contractions of 11.0 and 16.3 mm,

respectively. Strips from some aortas can respond fairly rapidly to

methoxamine (1×10^{-7}), but none of these is included in the results

presented in this section.

Strips contracted with either concentration of methoxamine
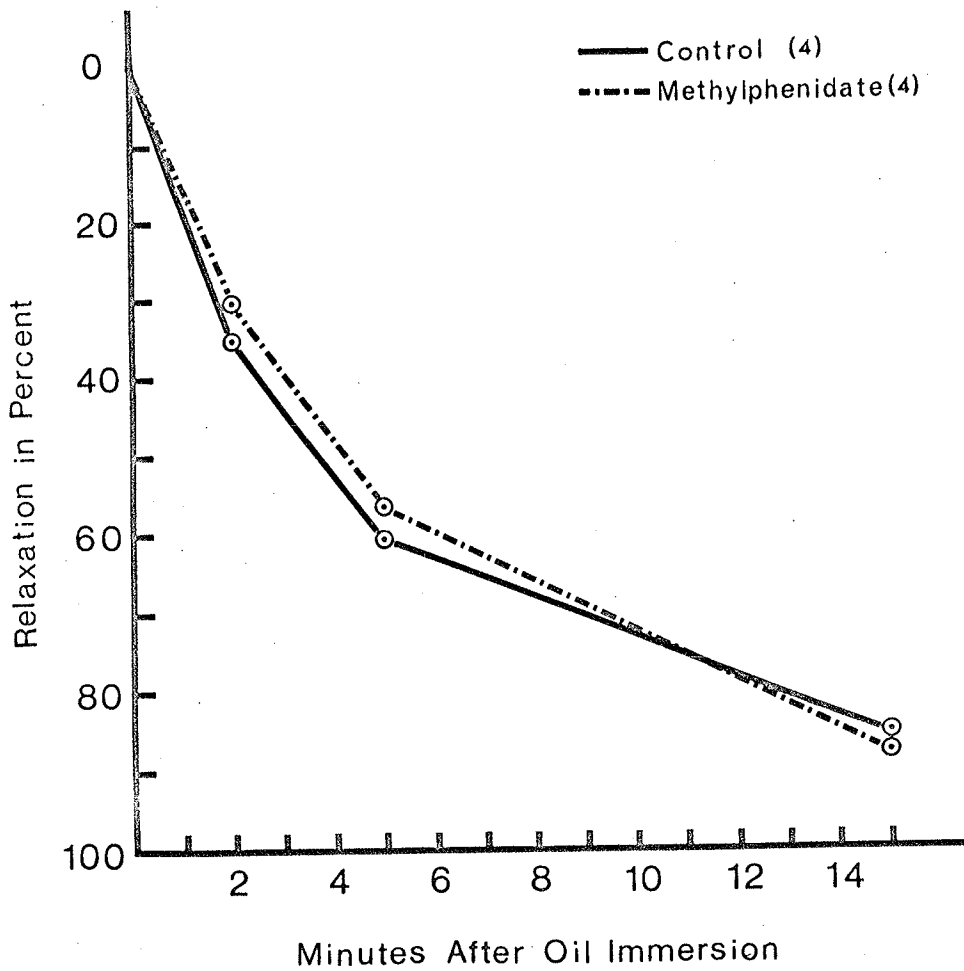
Fig. 36. Effect of Methylphenidate on the Relaxation of Histamine Contracted Aortic Strips after Oil Immersion.

Concentrations of histamine and methylphenidate were 1×10^{-6} and 1×10^{-5}, respectively. Figures in parentheses indiate number of preparations represented by each curve.

a.

b.
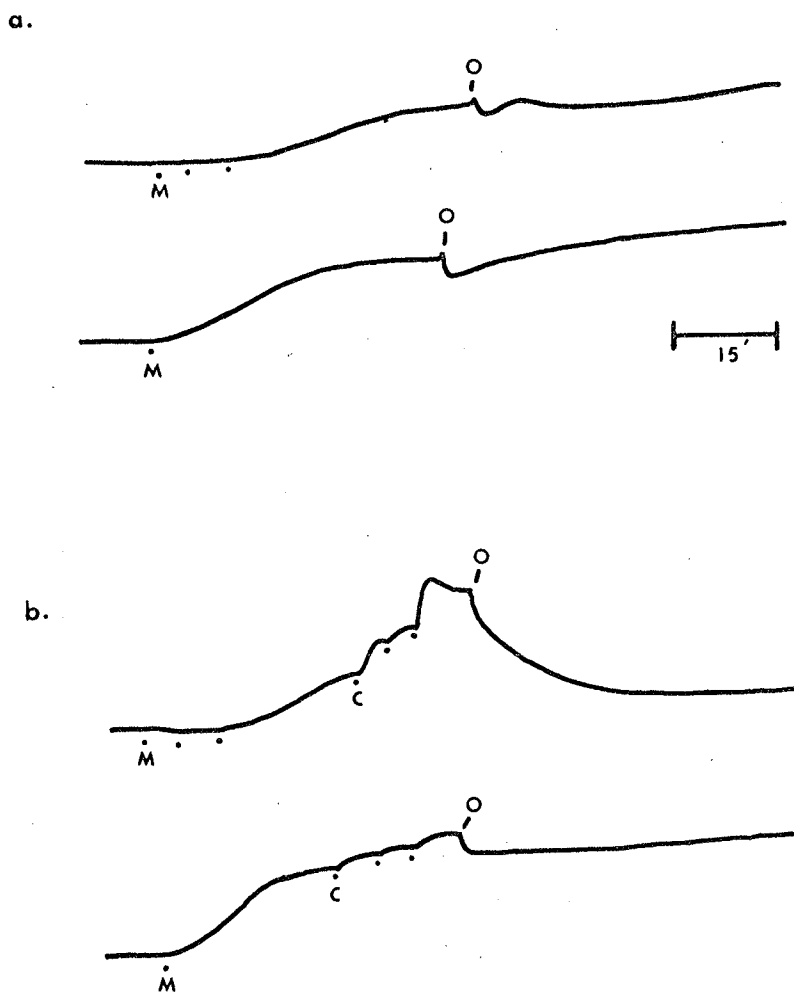
Fig. 37. Potentiation of Responses to Methoxamine by Cocaine.

   a. Responses to methoxamine (M), followed by oil immersion (O). Upper, cumulative concentrations of 1, 2 and 5×10^{-8} (dots); lower, single concentration of 1×10^{-7}.

b. Upper, effect of cumulative concentrations of cocaine (C) (1×10^{-7}, 1×10^{-6}, 1×10^{-5}) on a strip contracted by cumulative concentrations of methoxamine (1, 2 and 5×10^{-8}); lower, effect of the same concentrations of cocaine on a methoxamine (1×10^{-7}) contracted strip.

showed no evidence of drug inactivation during periods of oil immersion up to almost one hour (Fig. 34a). The initial brief relaxation after oil immersion seen in methoxamine contracted strips has also been observed with other agonists (e.g., phenylephrine) when major mechanisms for their inactivation were either blocked or absent. A methoxamine (1×10^{-7}) contracted strip washed in Krebs solution relaxed 59.5% in 15 minutes.

Two of the strips referred to above, contracted with methoxamine (5×10^{-8}), were treated with cumulative concentrations of cocaine (1×10^{-7}, 1×10^{-6}, 1×10^{-5}). (See Fig. 37b.) The increment in the responses of these strips produced by cocaine were 167% and 92% of the contraction produced by methoxamine alone. Figure 37b also shows the response to cocaine of a strip contracted with methoxamine (1×10^{-7}). In all cases the increase in contraction height due to cocaine occurred much more rapidly than did the initial contraction produced by methoxamine alone.

The potentiation of responses to methoxamine (5×10^{-8}) by cocaine (1×10^{-5}) was much greater than 2 times, in terms of equivalent concentrations of methoxamine, as can be seen by comparing the response to methoxamine (5×10^{-8}) plus cocaine with that to methoxamine (1×10^{-7}) alone (Fig. 37b).

When cocainized, methoxamine contracted strips were immersed in oil, the contractions declined to approximately the amplitude prior to the addition of cocaine and remained at this level for the duration of the period of observation, up to one hour.

6. Effect of Procaine on Responses to Phenylephrine and Noradrenaline:

The cumulative addition of procaine (1×10^{-7}, 1×10^{-6}, 1×10^{-5}) to the chambers of strips contracted by phenylephrine (3×10^{-8}) produced no observable effect except a slight depression of contraction amplitude at 1×10^{-5} (Fig. 38a). Procaine (1×10^{-5}) also did not potentiate contractions produced by noradrenaline (1×10^{-8}). However, procaine did significantly slow the relaxation of phenylephrine (3×10^{-8}) contracted strips in oil (Fig. 39). As in the case of cocaine, procaine essentially eliminated the relaxation in oil of phenylephrine contracted strips which had been pretreated with iproniazid (Fig. 38b).

Although procaine apparently shares with cocaine an action which impairs the inactivation of sympathomimetic amines, it appears to lack entirely the potentiating action of cocaine, and its presence does not alter potentiation by the latter agent (Fig. 38a).

7. Effect of Cocaine on Responses of Stored Aortic Strips to

Noradrenaline:

To provide additional evidence that the potentiation of responses to sympathomimetic amines by cocaine is not due to impairment of their uptake by nerves, the effects of cocaine were studied on aortic strips which had been stored for prolonged periods to permit degeneration of the severed sympathetic nerves.

Four strips cut from a single aorta were contracted with noradrenaline (1×10^{-8}) and exposed to cocaine (1×10^{-4}). After washout and recovery to basal tone, the strips were placed in individual vials containing oxygenated Krebs solution, sealed with parafilm and
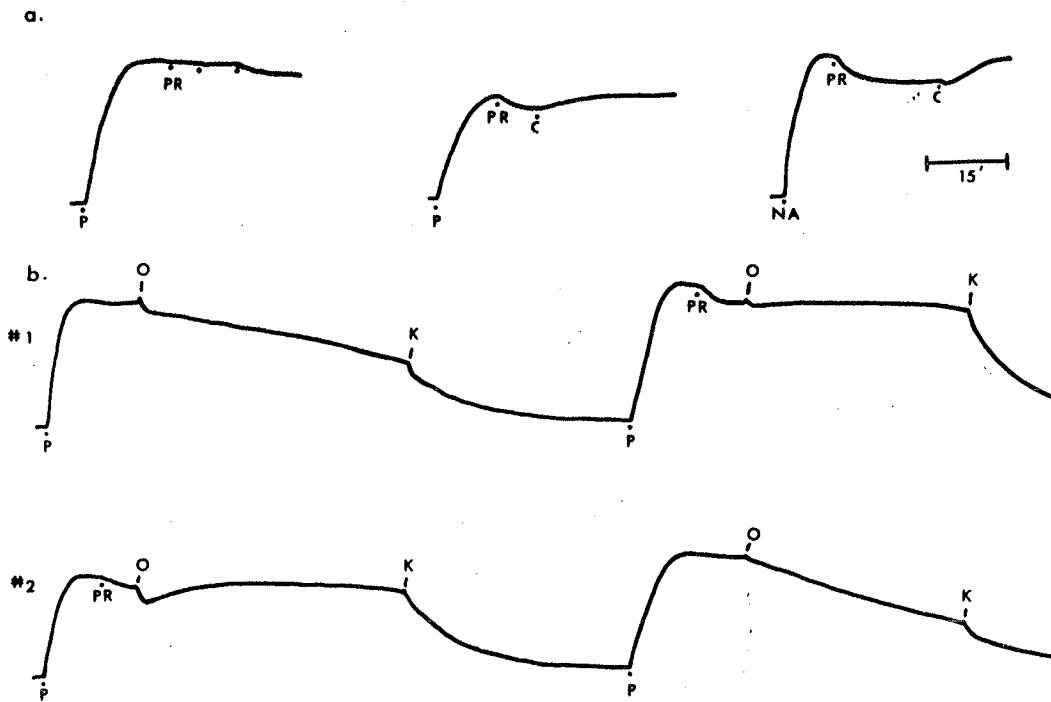
Fig. 38. Effects of Procaine and Cocaine on Noradrenaline and Phenylephrine Contracted Aortic Strips.

a. Left, response of phenylephrine (P) (3×10^{-8}) contracted strip to cumulative concentrations of procaine (PR) (1×10^{-7}, 1×10^{-6}, 1×10^{-5})(dots); center, response of phenylephrine contracted strip to procaine (1×10^{-5}), followed by cocaine (C) (1×10^{-5}); right, response of iproniazid pretreated noradrenaline (NA) (1×10^{-8}) contracted strip to procaine (1×10^{-5}), followed by cocaine (1×10^{-4}). b. Relaxation after oil immersion (O) of 2 iproniazid pretreated phenylephrine contracted strips in the presence and in the absence of procaine (1×10^{-5}). Return of strips to Krebs solution indicated by (K).
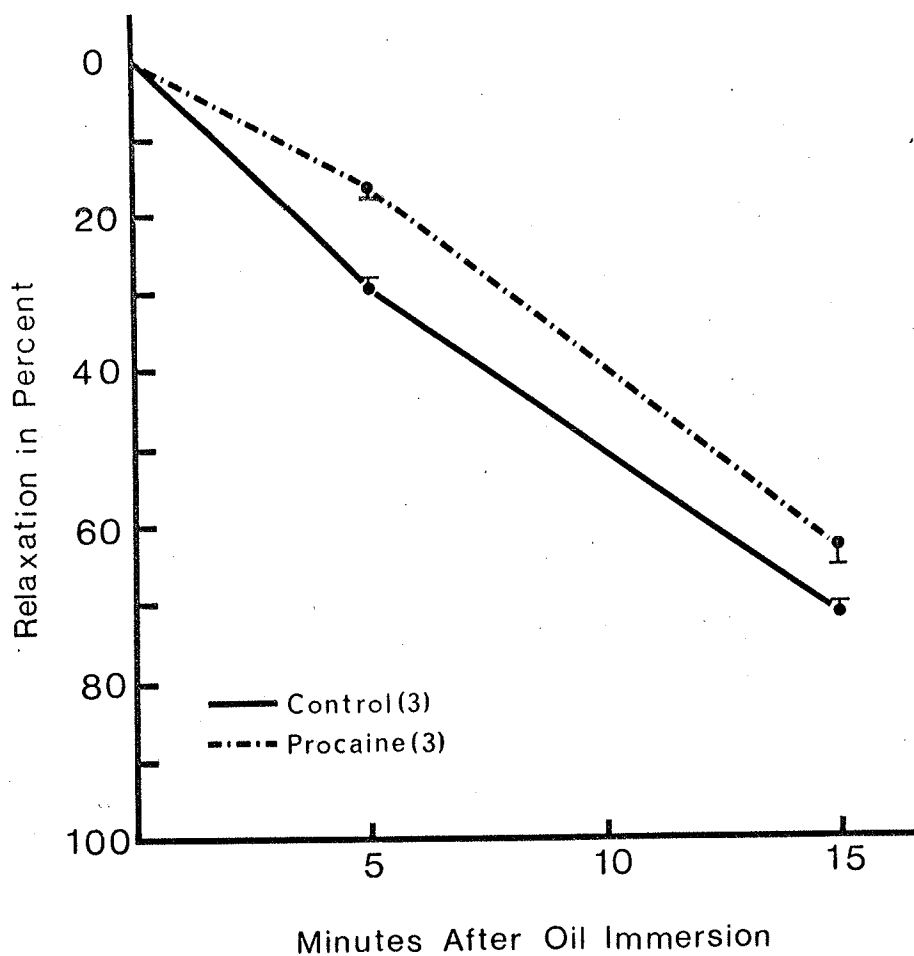
Fig. 39. Effect of Procaine on Relaxation of Phenylephrine Contracted Aortic Strips after Oil Immersion.

Phenylephrine and procaine concentrations were 3×10^{-8} and 1×10^{-5}, respectively. Figures in parentheses indicate number of preparations represented by each curve. Bars indicate standard errors of means.

Statistical analysis:

	Min.
Procaine vs. Control	5 - $P < 0.01$
	15 - $P < 0.05$

stored at 6°C. On the 5th day they were resuspended in Krebs solution

at room temperature and the chambers slowly raised to 37°C. After a

2- to 3-hour adjustment period, they were again contracted with

noradrenaline (1 X 10^{-8}) and exposed to cocaine (1 X 10^{-4}). This

procedure was repeated on the 8th, 11th and 16th days, except that 2 of

the strips were used for tests with tyramine rather than with noradren-

aline and cocaine. The results are presented in figure 40 and Table

XVIIIa. Augmentation of the noradrenaline contractions, expressed

either in mm or as percent of the contraction height prior to the

addition of cocaine, was not clearly decreased until after 10 days of

storage (Day 11). In fact, the cocaine increment was greater on the

5th than on the 1st day. To evaluate the condition of endogenous

catecholamine stores, strip #4, which had not previously been exposed

to tyramine, was tested with this agent on day 5, after pretreatment

with iproniazid to insure high sensitivity. Concentrations of tyramine

below 1 X 10^{-5} produced no response (Fig. 40); the small effect of

1 X 10^{-5} may reflect a direct action of tyramine on smooth muscle

receptors (Furchgott, 1955). Strip #3 was pretreated with iproniazid

and tested with tyramine on the 11th day. Cumulative administration of

tyramine (1 X 10^{-7}, 1 X 10^{-6}, 1 X 10^{-5}) produced no contraction in Krebs

solution or after oil immersion.

Changes in cocaine potentiation with time were also studied

in strips kept in the muscle chambers at 37°C, where degeneration of

intramural nerves should have been rapid. Control responses to noradren-

aline (1 X 10^{-8}) followed by cocaine (1 X 10^{-4}) were obtained about 4

hours after death of the animal. The chambers were then washed out,

and the strips retested about 22 1/2 hours and again 28 hours after
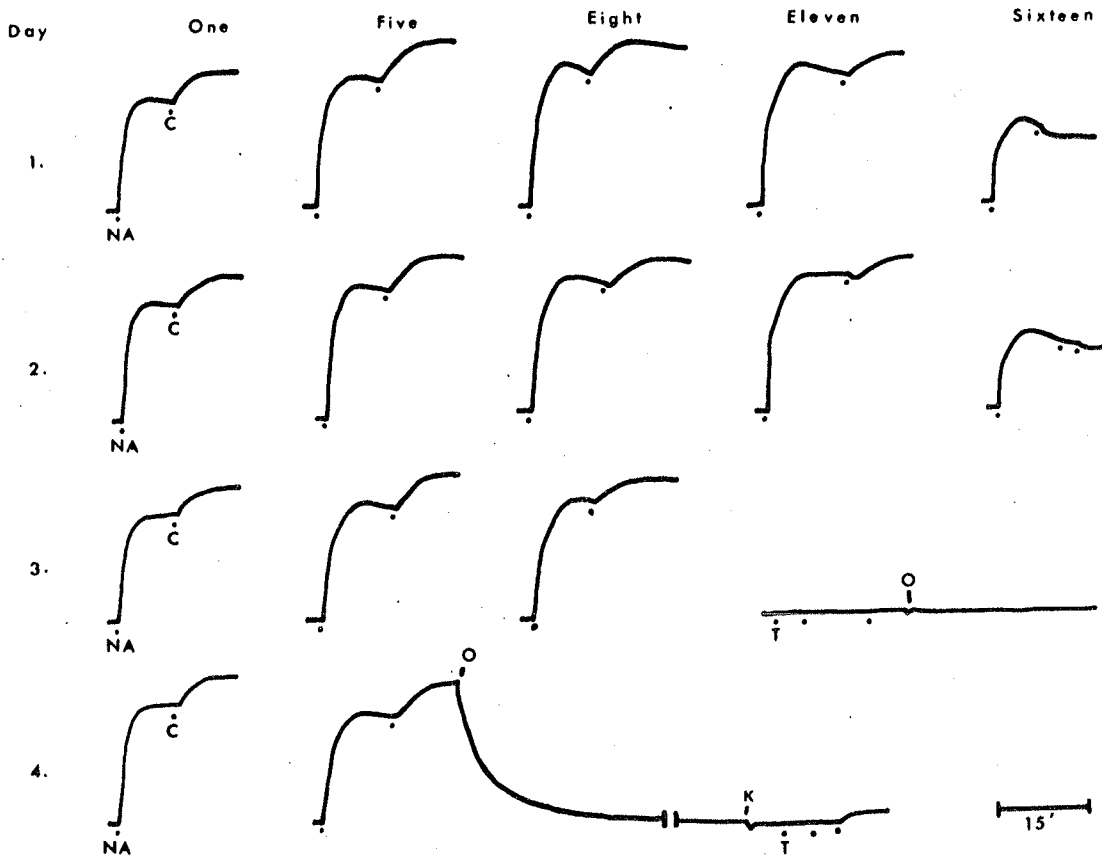
Fig. 40. Effect of Prolonged Storage in the Cold on Responses of Noradrenaline Contracted Aortic Strips to Cocaine.

Responses of 4 strips from the same aorta to noradrenaline (NA) (1×10^{-8}) and cocaine (C) (1×10^{-4}) on the indicated days of testing. All strips were stored at $6°C$ between tests. Strip #2 was exposed to cumulative concentrations of cocaine (1×10^{-5}, 1×10^{-4}) on day 16. Strip #4 was pretreated with iproniazid and tested to cumulative concentrations of tyramine (T), (1×10^{-7}, 1×10^{-6}, 1×10^{-5}) on day 5. Strip #3 was pretreated with iproniazid and tested similarly with tyramine on day 11. Oil immersion indicated by (O) and return to Krebs solution by (K).

TABLE XVIIIa

EFFECT OF INTERVAL AFTER AXOTOMY ON POTENTIATION BY

COCAINE OF RESPONSES OF AORTIC STRIPS TO NORADRENALINE

Strip #	Interval at 6°C (days)	Storage at 6°C Contraction Amplitude (mm)	Cocaine Increment mm	%
1	0	26	7	26.9
	4	31	9	29.0
	7	33	7	21.2
	10	31	5	16.1
	15	18	-3	-16.7
2	0	29	7	24.1
	4	31	8	25.8
	7	31	6	19.4
	10	33	4	12.1
	15	16	-2	-12.5
3	0	25	6	24.0
	4	27	8	29.6
	7	28	5	17.9
	10		tyramine test	
4	0	28	7	25.0
	4	26	8	30.8
	4		tyramine test	
x̄ 1 to 4	0	27.0	6.8	25.0
	4	28.8	8.3	28.8
	7	30.7	6.0	19.5
	10	32.0	4.5	14.1
	15	17.0	-2.5	-14.6

Strips were stored at 6°C except for about 4 hours at 37°C for each test. Values are given to the nearest half mm.

All test contractions were produced by noradrenaline (1×10^{-8}).

TABLE XVIIIb

EFFECT OF INTERVAL AFTER AXOTOMY ON POTENTIATION BY
COCAINE OF RESPONSES OF AORTIC STRIPS TO NORADRENALINE

Strip #	Interval at 37°C (hours)	Maintained at 37°C in Muscle Chambers Contraction Amplitude (mm)	Cocaine Increment mm	%
1	4	19	5	26.3
	22.5	14	3.5	25.0
	28*	5	2	40.0
2	4	18	6	33.3
	22.5	10	2	20.0
	28*	3	2	66.7
3	4	15	5	33.3
	22.5	8	2	25.0
4	4	18	4	22.2
	22.5	12	2	16.7
$\bar{x}$ 1 to 4	4	17.5	5	28.8
	22.5	11.0	2.4	21.7
	28	4	2	53.3

* Cocaine (1×10^{-5}), all others cocaine (1×10^{-4})

All test contractions were produced by noradrenaline (1×10^{-8})
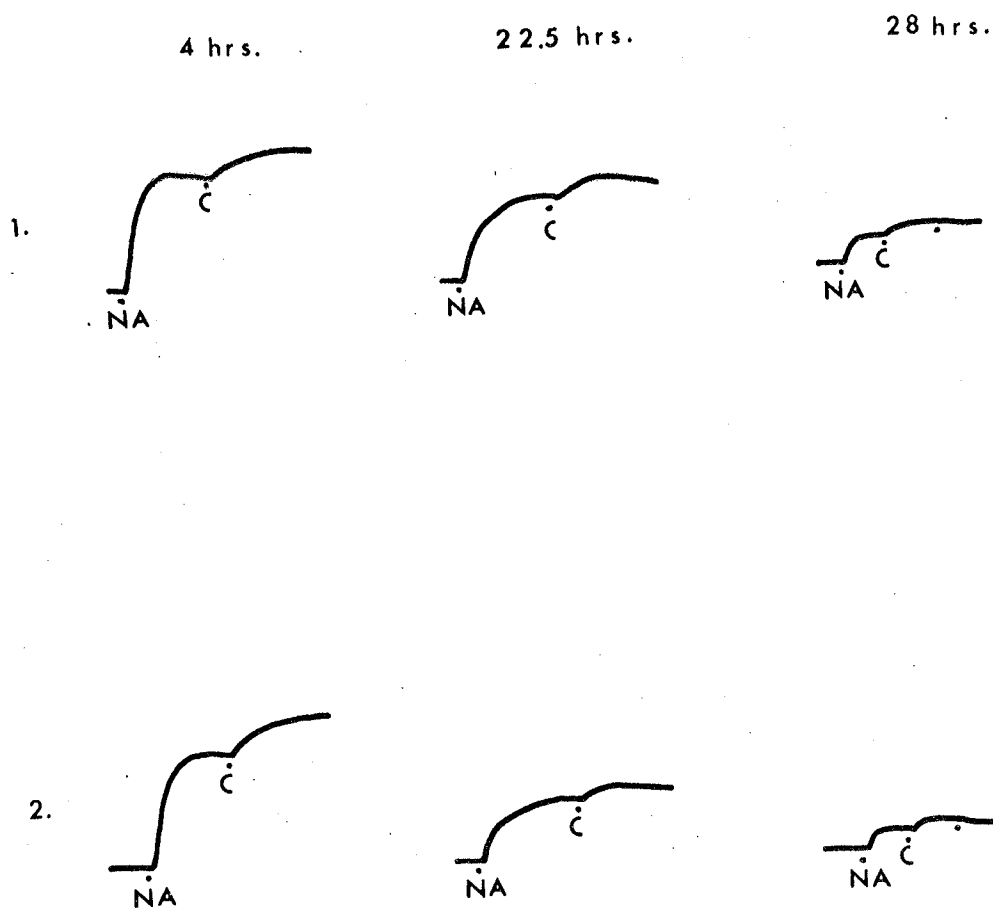
Fig. 41. Effect of Time at 37°C In Vitro on Responses of Noradrena-
line Contracted Aortic Strips to Cocaine.

Responses of 2 strips from the same aorta to noradrenaline
(1×10^{-8}) and cocaine (C) (1×10^{-4}) at the indicated times after
death of the animal. The test performed after 28 hours was with
cumulative concentrations of cocaine $(1 \times 10^{-5}, 1 \times 10^{-4})$. Strips
were kept in aerated Krebs solution in individual muscle chambers at
37°C throughout the experiment.

death of the animal (Fig. 41 and Table XVIIIb). Cocaine still

potentiated the responses 28 hours after preparation of the strips

(axotomy), although the diminished responses to noradrenaline indicated

that considerable muscle cell damage had occurred by this time.

B. RESULTS OF STUDIES ON THE EFFECTS OF GD-131 ON ADRENERGIC MECHANISMS

The work of Brown and Gillespie (1957), Brown (1960), Bacq

et al. (1960), Kirpekar and Cervoni (1963), Gillespie and Kirpekar

(1965), and others implicated the adrenergic receptors per se in the

termination of the action of noradrenaline, largely on the basis of

effects of the β-haloalkylamine α-adrenergic blocking agents,

Dibenamine and phenoxybenzamine, on the output of noradrenaline from

sympathetically innervated organs. The experiments reported in this

section were undertaken in an attempt to evaluate the contribution of

adrenergic receptors to the inactivation of sympathomimetic amines,

and to determine the relationship, if any, between the actions of the

β-haloalkylamines and those of cocaine.

The experiments employed the oil immersion technique to

measure the rate of inactivation of sympathomimetic amines in rabbit

aortic strips. GD-131 (N-cyclohexylmethyl-N-ethyl-β-chloroethylamine)

(Fig. 42c), was selected for study because it has the chemical

reactivity characteristic of the β-haloalkylamine group, comparable to

that of Dibenamine and phenoxybenzamine (Harvey and Nickerson, 1954),

but has very little α adrenergic blocking activity. It has been re-

ported to potentiate certain responses to catecholamines (Furchgott,

a.

b.

c.

$$Cl-CH_2-CH_2-N \begin{array}{c} \diagup C_2H_5 \\ \diagdown CH_2-\langle S \rangle \end{array}$$
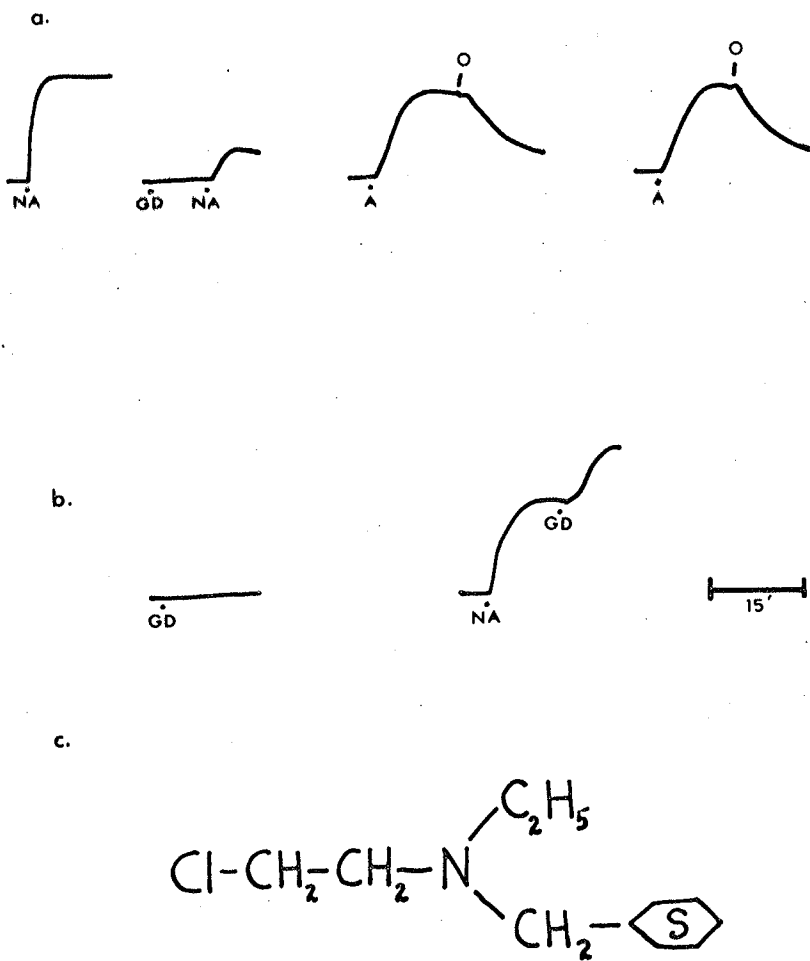
Fig. 42. Effects of GD-131 on the Basal Tone of Unreserpinized Aortic Strips and on Responses to Angiotensin and Noradrenaline.

a. Responses of a strip to noradrenaline (NA) (1 X 10^{-8}) (left) and to angiotensin (A) (3 X 10^{-9}) (right). In each pair, response on the left is before and that on the right after exposure to GD-131 (GD) (1 X 10^{-4} for 10 min.). Oil immersion indicated by (O). b. Response to GD-131 (1 X 10^{-5}) of a quiescent strip (left) and of a strip contracted with noradrenaline (right). All strips were from the same aorta. c. Chemical structure of GD-131.

1960 c). It was felt that these properties might allow detection of any distinction which might exist between effects on α adrenergic receptors and on mechanisms for inactivation of sympathomimetics and, thus, allow a more precise assessment of the contribution of the receptors to termination of action. GD-131 has also been reported to release catecholamines from the heart (Furchgott and Kirpekar, 1960) and, unless otherwise indicated, all experiments were done on aortic strips from reserpinized rabbits (5.0 mg/kg, intramuscularly, 16 to 24 hours before death) to minimize complications which might arise from endogenously released noradrenaline.

1.   Effects of GD-131 on Unreserpinized Aortic Strips:

A limited study was carried out on strips of aorta from un-reserpinized rabbits. A 15 minute exposure to a concentration of 1×10^{-5} GD-131 had little or no effect on the basal tone of the strips and markedly potentiated their responses to noradrenaline (Fig. 42b). Some unreserpinized strips exposed to GD-131 (1×10^{-5}) did contract after oil immersion. This was obviously due to magnification of the effect of very small amounts of catecholamine released from endogenous stores. However, even strips which did not shorten in oil after pretreatment with GD-131 (1×10^{-5} for 15 min.) relaxed considerably slower than did control strips after being contracted by noradrenaline (1×10^{-8}). This effect on amine disposition was studied more thoroughly on aortic strips from reserpinized rabbits.

Pretreatment of aortic strips for 10 minutes with a concentration of 1×10^{-4} GD-131 markedly depressed responses to noradrenaline, but had no apparent effect on the amplitude of responses of aortic

strips to angiotensin or on their subsequent relaxation in oil

(Fig. 42a). This differential indicated that appreciable α adrenergic

receptor blockade was produced by this concentration of the β-haloalkyl-

amine.

2.   Effects of GD-131 on Reserpinized Aortic Strips:

It quickly became apparent that, unlike those of cocaine,

certain actions of GD-131 were not readily reversible after washout of

the chambers. This demonstrated a further similarity of the actions of

GD-131 and those of Dibenamine and phenoxybenzamine. For example,

GD-131 (1×10^{-5}), added 15 minutes before oil immersion, considerably

potentiated the response of a noradrenaline (1×10^{-8}) contracted strip,

and slowed its subsequent relaxation in oil (Fig. 43a, left). The same

strip was recontracted with noradrenaline (1×10^{-8}) after washout and

recovery from the first test (Fig. 43a, right). A second equal

exposure to GD-131 produced a barely detectable increase in the

amplitude of contraction, although a further decrease in the rate of

relaxation is apparent. In association with other results with GD-131,

it has become clear that these characteristics of the second exposure

to the drug are determined by persistence of changes produced by the

first.

The effect of a relatively long exposure of a noradrenaline

(1×10^{-8}) contracted strip to GD-131 (1×10^{-5} for 25 min.) is shown

in figure 43b (right), and the control response of the same strip to

noradrenaline (left). It appears that the action of GD-131 (1×10^{-5})

which potentiates responses to noradrenaline is exerted rapidly, like

that of cocaine, but that the action which decreases the rate of
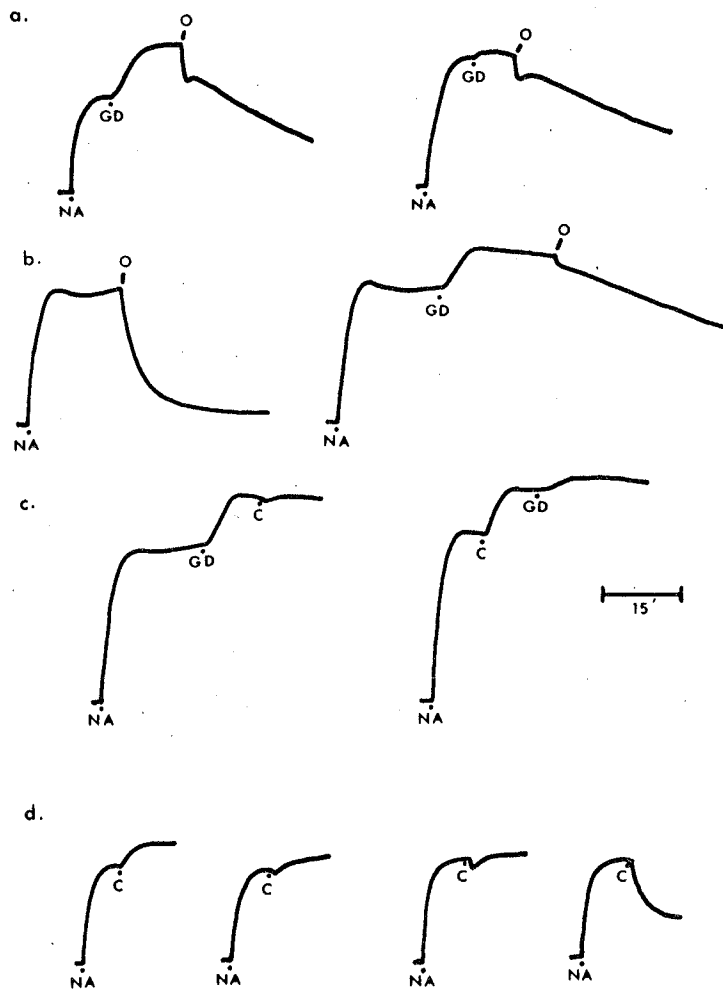
Fig. 43. Effects of Cocaine and GD-131 on Noradrenaline Contracted Aortic Strips.

a. Responses of an aortic strip in Krebs solution and after oil immersion to a first (left) and second (right) exposure to GD-131 (GD) (1×10^{-5}) after contractions produced by noradrenaline (NA) (1×10^{-8}). b. Left, response to noradrenaline and subsequent relaxation after oil immersion; right, same strip recontracted with noradrenaline and exposed to GD-131 (1×10^{-5}) for 25 min. before oil immersion. c. Left, effect of cocaine (C) (1×10^{-4}) on a noradrenaline contracted strip after exposure to GD-131 (1×10^{-5}); right, the effect of GD-131 (1×10^{-5}) on a noradrenaline contracted strip after exposure to cocaine (1×10^{-4}). d. Responses of noradrenaline contracted strips to various concentrations of cocaine, from left to right, 2, 3 and 5×10^{-4}, 1×10^{-3}.

relaxation develops more slowly during exposure to the drug.

3. <u>Mechanisms by which Cocaine and GD-131 Potentiate Responses to</u>

   <u>Noradrenaline</u>:

   When noradrenaline (1×10^{-8}) contracted aortic strips were

exposed to cocaine (1×10^{-4}) and to GD-131 (1×10^{-5}) in sequence,

cocaine added after GD-131 caused only a barely detectable potentiation,

whereas GD-131 exerted a reduced, but much more definite effect after

cocaine (Fig. 43c). The results of a series of such tests are presented

in Table XIXa. In agreement with earlier results, cocaine (1×10^{-4})

caused a greater potentiation of responses to noradrenaline than did

cocaine (1×10^{-5}). Although the former concentration was shown to

produce the maximal potentiation possible with this agent (Fig. 43d),

GD-131 (1×10^{-5}) caused a significantly greater potentiation than did

either dose of cocaine.

4. <u>Effects of GD-131 on Responses to Tyramine</u>:

   The interaction of GD-131 and tyramine was studied on aortic

strips from unreserpinized rabbits because reserpine pretreatment

markedly depresses responses to this agonist. In contrast to its

potentiation of responses to noradrenaline, cocaine markedly depresses

those to tyramine, presumably by blocking the nerve membrane transport

mechanism through which tyramine gains access to stores of catechol-

amine. (See Historical Introduction.)

   Figure 44a shows records from an experiment on 2 strips

from a single aorta pretreated with iproniazid and contracted with

tyramine. One was exposed to cocaine and the other to GD-131 in

cumulatively increasing concentrations. Cocaine progressively

a.

b.

c.
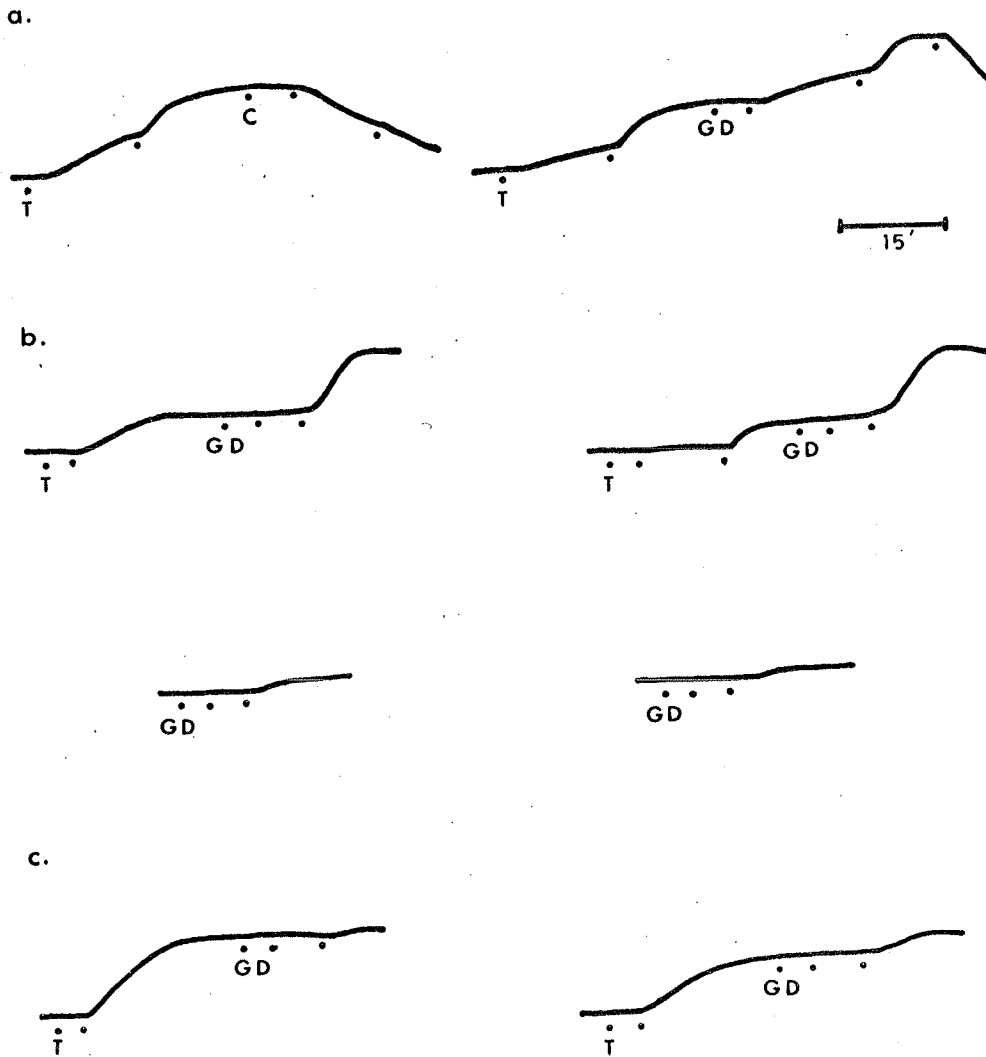
Fig. 44. Effects of GD-131 and Cocaine on Tyramine Contracted Aortic
Strips.

a. Responses of 2 iproniazid pretreated strips from the same
aorta to tyramine (T) (1 and 3 X 10^{-7}), followed by cocaine (C) (1 X 10^{-7},
1 X 10^{-6}, 1 X 10^{-5}) (left) or GD-131 (GD) (1 X 10^{-7}, 1 X 10^{-6}, 1 X 10^{-5},
1 X 10^{-4}). b. Responses of 4 iproniazid pretreated strips to cumulative
concentrations of GD-131 (1 X 10^{-7}, 1 X 10^{-6}, 1 X 10^{-5}) alone (lower) or
after contraction by tyramine (left, 1 and 3 X 10^{-7}; right, 1 and 3 X 10^{-7},
1 X 10^{-6}). c. Responses of 2 iproniazid plus tropolone pretreated
strips to tyramine (1 and 3 X 10^{-7}), followed by GD-131 (1 X 10^{-7},
1 X 10^{-6}, 1 X 10^{-5}). All strips in b and c were from the same aorta.

depressed the tyramine response, as expected, but GD-131 increased it. Depression by the GD-131 occurred only at a concentration of 1×10^{-4}, which was previously shown to produce considerable α adrenergic blockade.

The contribution of the known catecholamine releasing action of GD-131 to its potentiation of responses to tyramine was evaluated on strips from an unreserpinized aorta pretreated with iproniazid. Two strips were contracted with tyramine before addition of GD-131 and 2 were exposed to only the β-haloalkylamine. The effect of endogenous catecholamine released by GD-131 is obviously inadequate to account for the potentiation of responses to tyramine (Fig. 44b). In fact, this probably would make a negligible contribution to the amplitude of the responses of strips already contracted by tyramine.

The effect of pretreatment with tropolone on the potentiation of responses to tyramine by GD-131 is shown in figure 44c; the 2 strips were cut from the same aorta as those whose responses are shown in panel b of this figure and all were pretreated with iproniazid. Although some potentiation was still apparent, it was markedly reduced in the tropolone pretreated strips.

5.   Effects of GD-131 on Responses to Methoxamine:

Experiments similar to those carried out to explore the effects of cocaine on responses to methoxamine (Fig. 37), and on strips from the same aorta, were performed with GD-131. Administered cumulatively (1×10^{-7}, 1×10^{-6}, 1×10^{-5}) after contractions produced by methoxamine had reached a plateau, GD-131 caused marked and progressive potentiation (Fig. 45a), similar to that produced by

a.

b.
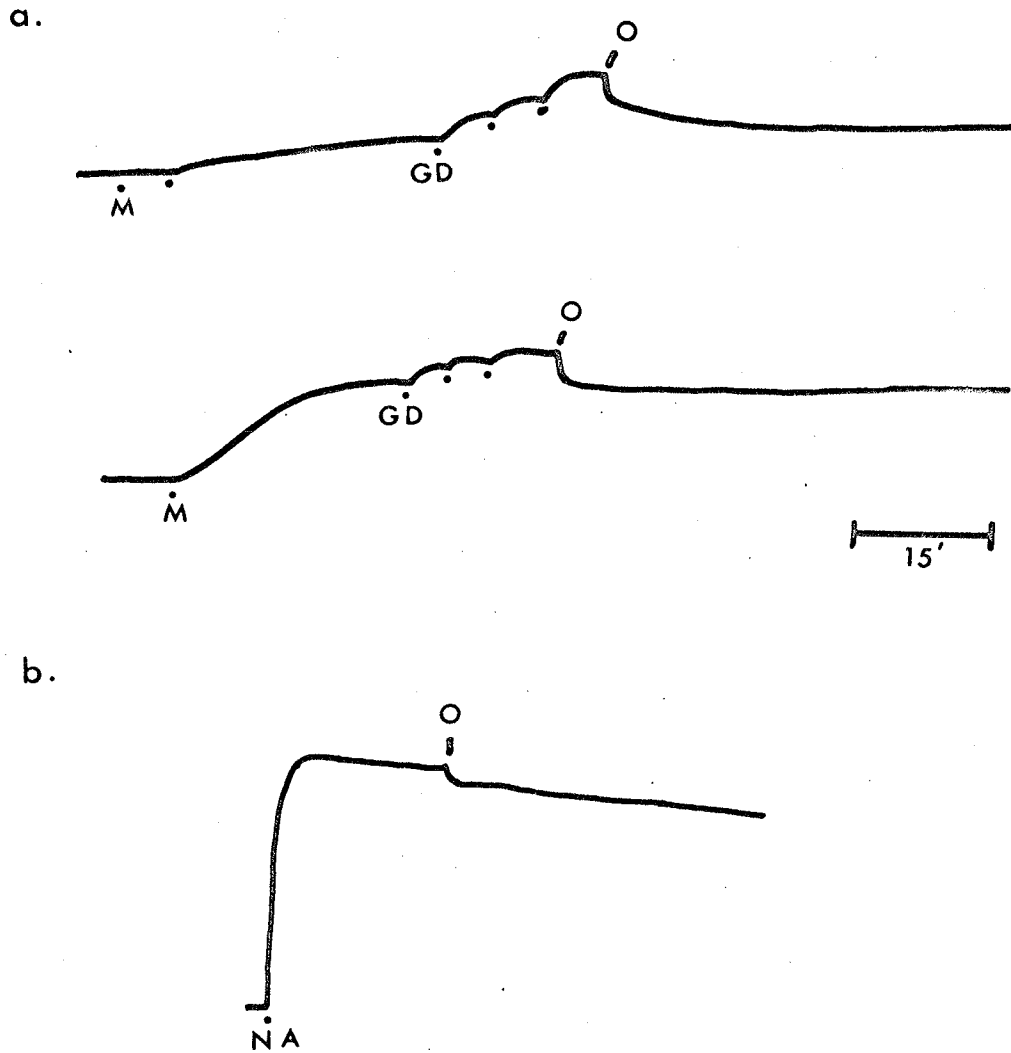
Fig. 45. Effects of GD-131 on Methoxamine and Noradrenaline Contracted Aortic Strips.

a. Responses of strips contracted by methoxamine (M) (2 and 5×10^{-8}) (upper) and by methoxamine (1×10^{-7}) (lower) to cumulative concentrations of GD-131 (GD) (1×10^{-7}, 1×10^{-6}, 1×10^{-5}). b. Response to noradrenaline (NA) (1×10^{-8}) and subsequent relaxation in oil (O) of a strip pretreated with GD-131 (1×10^{-5} for 30 min.).

cocaine. Methoxamine contracted strips treated with GD-131 also
responded to oil immersion in the same way as did cocaine treated
strips; contraction amplitude dropped rapidly to near the level prior
to the addition of GD-131 and then remained relatively constant.

6.   Effects of Cocaine and of GD-131 on the Relaxation of Noradrenaline

      Contracted Aortic Strips:

The course of relaxation after oil immersion (Fig. 46) was
studied on some of the same aortic strips on which potentiation of
noradrenaline responses was measured. Increasing the concentration of
cocaine 10 times, from 1×10^{-5} to 1×10^{-4}, did not produce a major
increase in the inhibition of rate of relaxation, indicating that
nearly the full effect of this agent had been achieved. GD-131
(1×10^{-5}) added to the chambers after the noradrenaline (1×10^{-8})
contractions had reached a plateau, 10 to 15 minutes before oil
immersion, slowed the relaxation distinctly more than did cocaine
(1×10^{-4}). Relaxation was almost completely prevented in 4 nor-
adrenaline contracted strips treated sequentially with both cocaine
(1×10^{-4}) and GD-131 (1×10^{-5}); in 3/4, the GD-131 was given first.

The effect of GD-131 on the rate of inactivation of noradren-
aline and its relationship to that of cocaine were studied in a
separate series of experiments in which GD-131 (1×10^{-5}) was added to
chambers containing quiescent strips and washed out after 10 minutes,
instead of being added after the strips had been contracted by nor-
adrenaline. The strips were washed at frequent intervals for 30
minutes and then contracted by noradrenaline (1×10^{-8}). Some of these
strips were also exposed to cocaine (1×10^{-4}) after the noradrenaline

Fig. 46. Effects of GD-131 and Cocaine on the Relaxation of Noradrena-
line Contracted Aortic Strips after Oil Immersion.

Cocaine and GD-131 were added after responses to noradrenaline
(1×10^{-8}) had reached a plateau, 10 to 15 minutes before oil immersion.
Strips treated with both cocaine and GD-131 received the drugs sequen-
tially. All strips were from animals pretreated with reserpine. Figures
in parentheses indicate number of preparations represented by each curve.
Bars indicate standard errors of means. Values for cocaine 1×10^{-5} did
not differ significantly at any time ($P < 0.2 > 0.1$, at 5 and 15 min.)
from those of cocaine (1×10^{-4}.

contractions had reached a plateau. Other preparations from the same aortas were contracted with noradrenaline without GD-131 pretreatment, and some of these were then exposed to cocaine (1×10^{-4}). The relaxation of all strips in oil was recorded. Records from 2 of these experiments are shown in figure 47, and the results of all 4 are graphed in figure 48 and tabulated in Table XIXb.

A 10 minute exposure of quiescent strips to GD-131 produced less inhibition of relaxation relative to the controls than did GD-131 added to the chambers 10 minutes before oil immersion. The difference is undoubtedly due to the continued action of GD-131 trapped in the tissue during the period of oil immersion. Strips treated with cocaine (1×10^{-4}) relaxed in oil at a rate not significantly different from that of strips pretreated with GD-131. However, relaxation was almost completely prevented by combined pretreatment with GD-131 (1×10^{-5}) and exposure to cocaine (1×10^{-4}).

Pretreatment with GD-131 (1×10^{-5}) for 30 minutes inhibited the relaxation of noradrenaline contracted strips considerably more than did a 10 minute exposure. The mean relaxation of 3 strips so treated was only 33.2% in 30 minutes of oil immersion, and one of these relaxed only 18.9% in 30 minutes (Fig. 45b). Although not consistently obtained by pretreatment with GD-131 alone, this rate of relaxation was similar to that of strips treated with the combination of iproniazid, tropolone and cocaine (1×10^{-4}). The development of α adrenergic blockade prevented exposure of aortic strips to GD-131 (1×10^{-5}) for periods of longer than 30 minutes. Prolonging the exposure to cocaine did not increase the inhibition of relaxation in oil which it produced (Fig. 23).
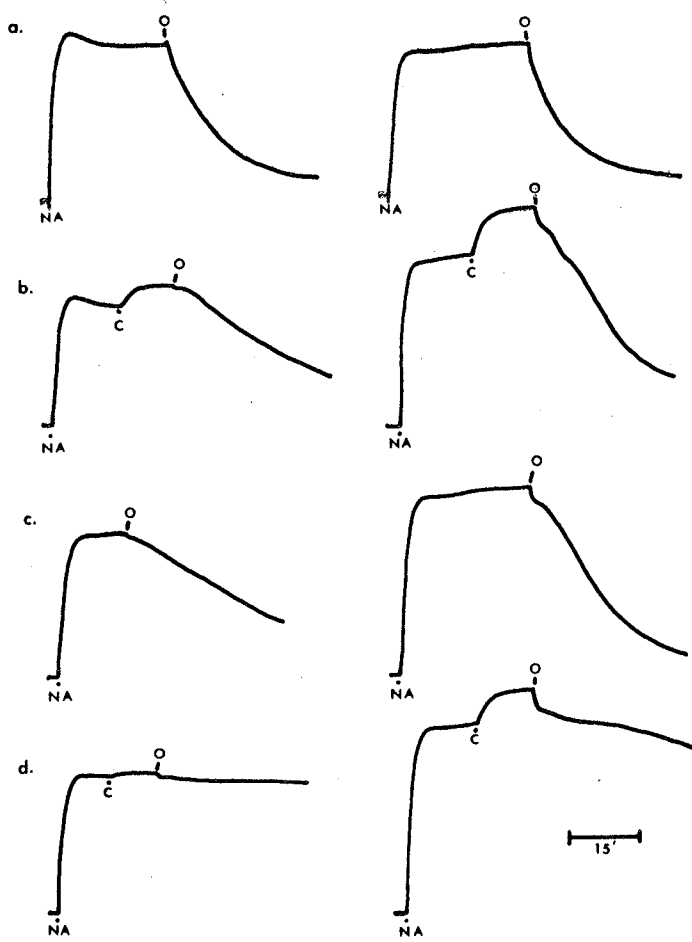
Fig. 47. Effects of GD-131 Pretreatment on the Relaxation of Cocaine Treated Noradrenaline Contracted Aortic Strips after Oil Immersion.

Records from 2 experiments are shown (left and right). All strips were contracted with noradrenaline (NA) (1×10^{-8}). a. Control responses, b. cocaine (C) (1×10^{-4}) added 10 minutes before oil immersion (O), c. pretreated with GD-131 (1×10^{-5} for 10 min.) 30 minutes before noradrenaline, d. pretreated with GD-131 as in c and exposed to cocaine as in b.
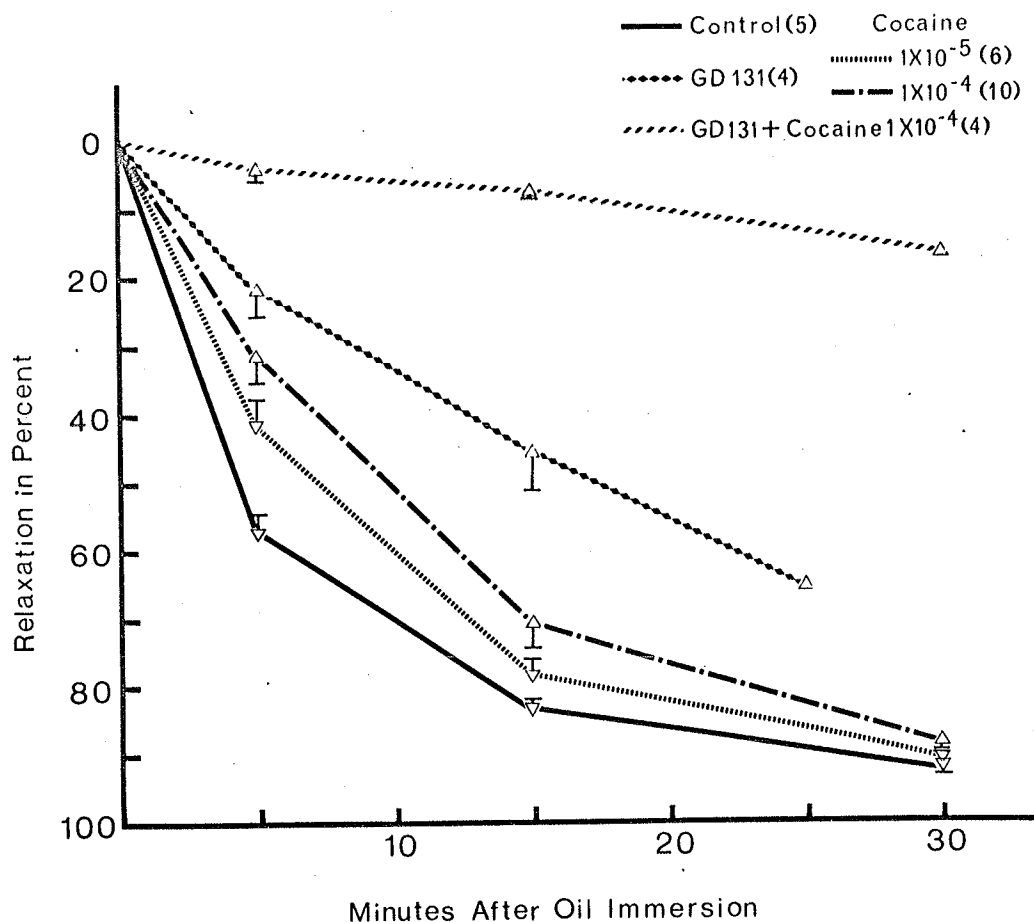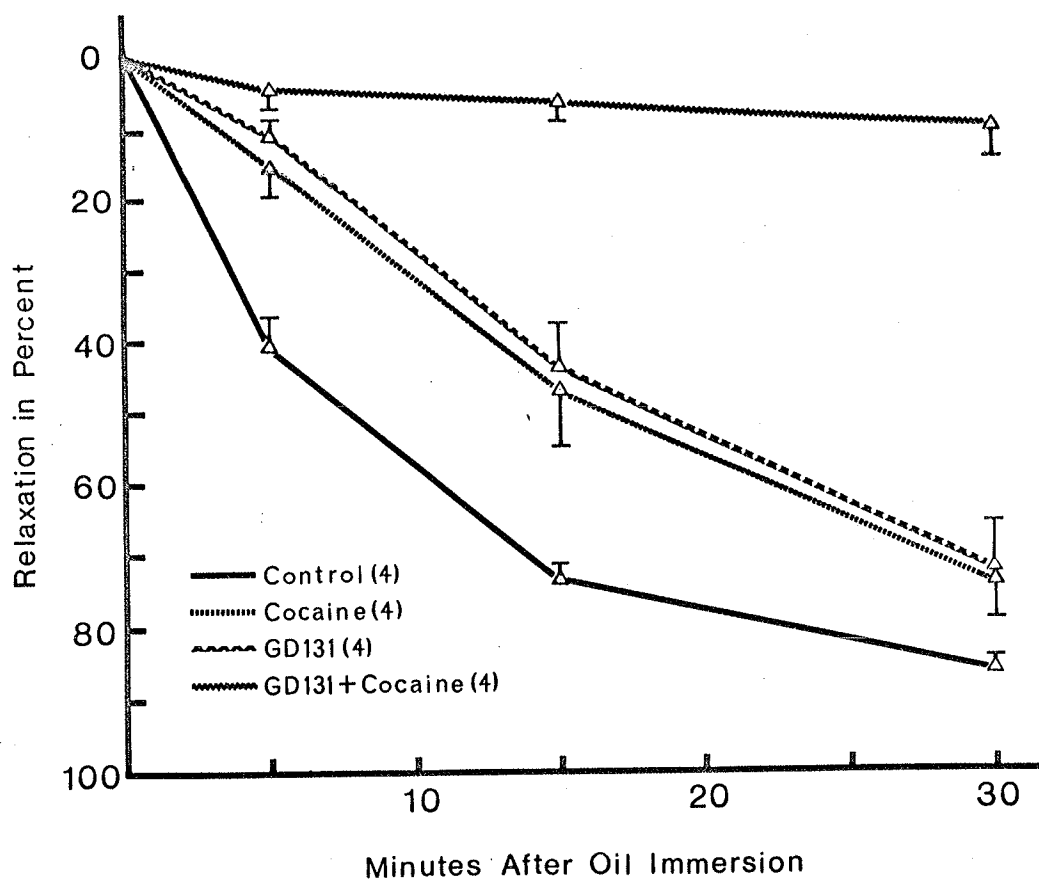
Fig. 48. Effects of GD-131 and Cocaine on Relaxation of Noradrenaline Contracted Aortic Strips after Oil Immersion.

Curves represent the means of 4 complete experiments, the records from 2 of which are shown in figure 47. All strips were contracted with noradrenaline (1×10^{-8}). Treatment with GD-131 (1×10^{-5} for 10 min.) was 30 minutes before noradrenaline; cocaine (1×10^{-4}) was added after noradrenaline contraction and 10 minutes before oil immersion. Bars indicate standard errors of means.

TABLE XIX

POTENTIATION BY COCAINE AND GD-131 OF RESPONSES

OF AORTIC STRIPS TO NORADRENALINE

a. The Effect of Supraddition of Cocaine and GD-131 on the Magnitude of Response of Noradrenaline (1×10^{-8}) Contracted Strips.

Drug(s)	No. Strips	Increment		Final Equivalent Conc. N.A.
		mm	%	
Cocaine (1×10^{-5})	6	8.8	21.4 ± 2.5	2.5×10^{-8}
Cocaine (1×10^{-4})	10	11.8	28.2 ± 2.0	4.0×10^{-8}
GD-131 (1×10^{-5})	8	13.5	37.1 ± 3.9	7.0×10^{-8}
Cocaine (1×10^{-4}) after GD-131 (1×10^{-5})	3	1.2	2.4 ± 0.5	8.5×10^{-8}
GD-131 (1×10^{-5}) after Cocaine (1×10^{-4})	1	4.0	6.9	8.0×10^{-8}

b. The Effect of Pretreatment with GD-131, (1×10^{-5}) for 10' on Cocaine Potentiation and Impairment of Inactivation of Noradrenaline (1×10^{-8}) Contracted Strips.

Drug(s)	No. Strips	Increment		Relaxation (X control)	NA normally in-activ. by mech. blocked (%)
		mm	%		
Cocaine (1×10^{-4})	4	8.5	17.9 ± 2.4	2.13	50 - 55
GD-131 (1×10^{-5})	4			2.36	55 - 60
Cocaine (1×10^{-4}) after GD-131 (1×10^{-5})	4	4.0	7.9 ± 3.3	24.0*	95

a. Drugs added after noradrenaline (NA) contraction reached plateau.

b. GD-131 pretreatment for 10 min. Cocaine added after noradrenaline contraction reached plateau. Only cocaine potentiation is given.

* Indicates relaxation compared to control at 10% rather than 50%.

Pretreatment with GD-131 (1×10^{-5}) for 10 minutes markedly

reduced the potentiation of subsequent noradrenaline contractions

caused by cocaine (Table XIXb). Responses of unpretreated strips were

potentiated 17.9% and those of strips pretreated with GD-131, only 7.9%

by cocaine (1×10^{-4}).

7. Effect of GD-131 Alone on Aortic Strips:

Unless otherwise indicated, all aortic strips used in these

experiments were from reserpinized animals. However, release of

endogenous catecholamine by GD-131 was not completely eliminated by

this procedure. Some strips exposed to GD-131 (1×10^{-5}) showed a sub-

sequent gradual increase in tone during oil immersion. This occurred

both in experiments in which the GD-131 was left in the chamber and

those in which it was washed out before oil immersion, and was un-

influenced by cocaine.

This release of endogenous catecholamine was quantitated in

7 strips pretreated for 10 minutes with GD-131 (1×10^{-5}), which was

washed out of some of the chambers before oil immersion. The strips

contracted a mean of 1.8 mm in 15 and of 5.2 mm in 30 minutes of oil

immersion. The concentration of noradrenaline required to produce a

5.2 mm contraction of reserpinized aortic strips was estimated from

separately determined dose-response curves to be about 4×10^{-10}.

This was negligible in terms of either the initial concentration of

noradrenaline (1×10^{-8}) or of that present in noradrenaline contracted

strips during the period of oil immersion. The concentration of

catecholamine resulting from release of GD-131 during 30 minutes of oil

immersion was inadequate even to produce a detectable change in the

relaxation in oil of contractions produced by agents which act through

receptors other than the α adrenergic receptors.

8. Effects of GD-131 on Histamine Contracted Aortic Strips:

Pretreatment of reserpinized aortic strips with GD-131

(1×10^{-5}) markedly depressed subsequent responses to histamine, and

GD-131 (1×10^{-5}) added to the chambers after strips contracted with

histamine (3×10^{-6}) had reached a plateau and about 10 minutes before

oil immersion produced either a slight reduction or no change in the

amplitude of contraction. Control histamine contracted strips relaxed

32.8 ± 0.5 and 76.6% and GD-131 pretreated strips 46.7 ± 4.9 and 79.8%,

respectively, during 5 and 15 minutes of oil immersion. Mean relaxation

curves for these strips and for 2 histamine contracted strips from the

same aorta treated with cocaine (1×10^{-4}) are shown in figure 49.

Neither GD-131 nor cocaine had a significant effect on the relaxation

of histamine contracted strips in oil. The tendency to a more rapid

relaxation of the GD-131 pretreated strips may reflect some blockade

of histamine receptors.

9. Effects of GD-131 on the Relaxation of Noradrenaline Contracted

Strips after Enzyme Inhibition:

The effect of GD-131 on relaxation in oil of noradrenaline

(1×10^{-8}) contracted strips pretreated with tropolone is shown in

figure 50. Records on the left are of strips treated with tropolone

only, and those on the right of tropolone pretreated strips exposed to

GD-131 (1×10^{-5}) for 15 minutes before oil immersion. Tropolone was

readded before the second contraction shown in panel a, but was washed

out and not readded prior to the second contraction in b. It is
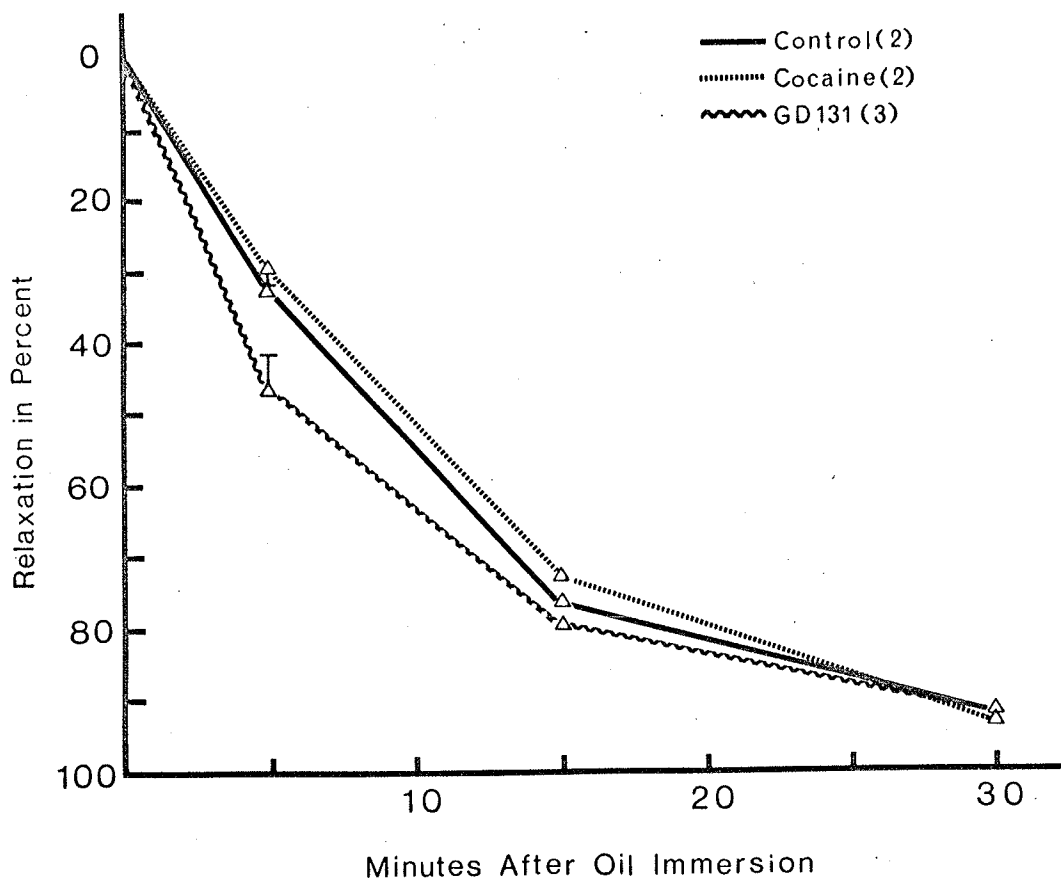
Fig. 49. Effects of GD-131 and Cocaine on the Relaxation of Histamine Contracted Aortic Strips after Oil Immersion.

All strips were contracted by histamine (3×10^{-6}); cocaine (1×10^{-4}) and GD-131 (1×10^{-5}) were added about 10 minutes before oil immersion. Figures in parentheses indicate number of preparations represented by each curve. Bars indicate standard errors of means.
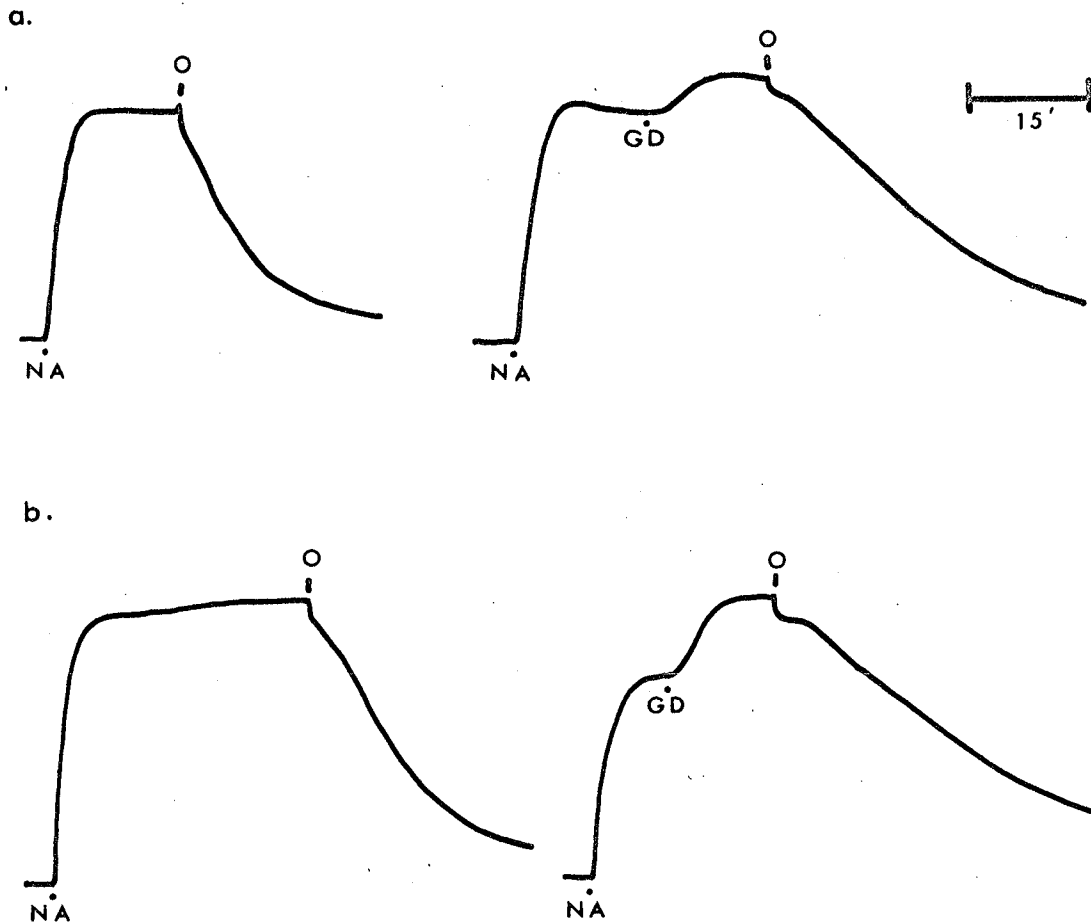
Fig. 50. Effects of Tropolone and GD-131 on Noradrenaline Contracted
Aortic Strips.

All strips were contracted by noradrenaline (NA) (1×10^{-8}),
followed by oil immersion (O). a. Two responses of the same strip to
noradrenaline (NA) (1×10^{-8}) in the presence of tropolone, second
contraction (right) followed by GD-131 (GD) (1×10^{-5}) 15 minutes before
oil immersion. b. Left, response to noradrenaline in the presence of
tropolone; right, response of same strip, after washout of tropolone,
to noradrenaline, followed by GD-131 (1×10^{-5}) 15 minutes before oil
immersion.

apparent from these records that GD-131 slows relaxation to a greater

extent than does inhibition of COMT. This is also demonstrated by the

fact that a 10 to 15 minute exposure to GD-131 (1 X 10^{-5}) and pre-

treatment with tropolone increased the time for 50% relaxation of

noradrenaline contracted strips to about 4.1 and 2.1 times that of

their controls, respectively (Fig. 46, Table XV).

Five strips pretreated with both iproniazid and tropolone

were contracted with noradrenaline (1 X 10^{-8}) and their relaxation

during 30 minutes of oil immersion recorded. After return to Krebs

solution and recovery of basal tone, these strips were again treated

with tropolone and recontracted with noradrenaline. Two of the strips

were exposed to cocaine (1 X 10^{-4}) for 10 minutes before and the re-

maining 3 to GD-131 (1 X 10^{-5}) for 10 to 15 minutes before oil immersion.

Records of the responses of 2 of these strips are shown in figure 51,

and the results of the series in figure 52.

As observed previously, cocaine (1 X 10^{-4}) significantly

slowed the rate of relaxation of aortic strips after inhibition of both

COMT and MAO. However, GD-131 had no significant effect on the rate of

relaxation after inhibition of both enzymes.

10. <u>Attempted Protection against Actions of GD-131 by Cocaine:</u>

GD-131 is a β-haloalkylamine whose action is not reversed by

washing tissues exposed to it. Consequently, it appeared suitable for

receptor protection experiments of the general type described in

Section III (A, 1, d). An attempt was made to determine if either the

potentiation of responses to noradrenaline or the inhibition of its

inactivation by GD-131 involved the same sites as the similar effects

a.

b.
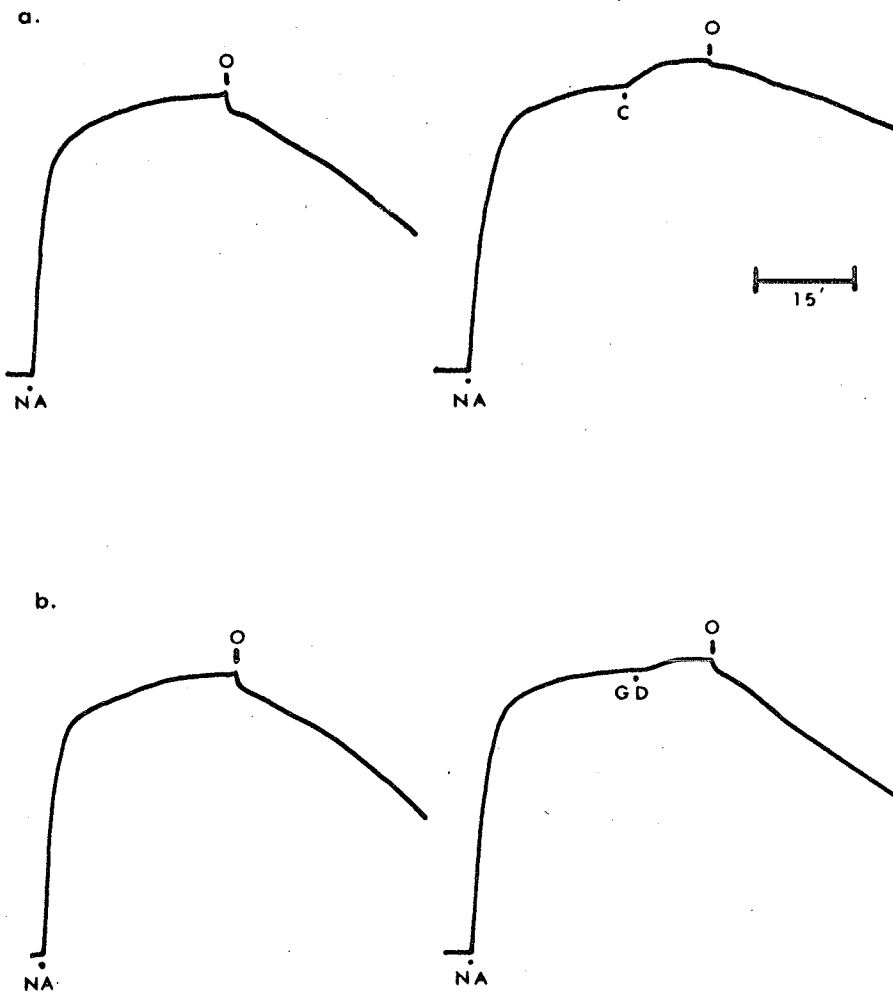
Fig. 51. Effects of GD-131 and Cocaine on Noradrenaline Contracted Aortic Strips after Enzyme Inhibition.

All strips were pretreated with iproniazid and tropolone, contracted by noradrenaline (NA) (1×10^{-8}) and allowed to relax in oil (O). a. Left, enzyme inhibition only; right, same strip with cocaine (C) (1×10^{-4}) added 10 minutes before oil immersion. b. Left, enzyme inhibition only; right, same strip with GD-131 (GD) (1×10^{-5}) added 10 minutes before oil immersion.
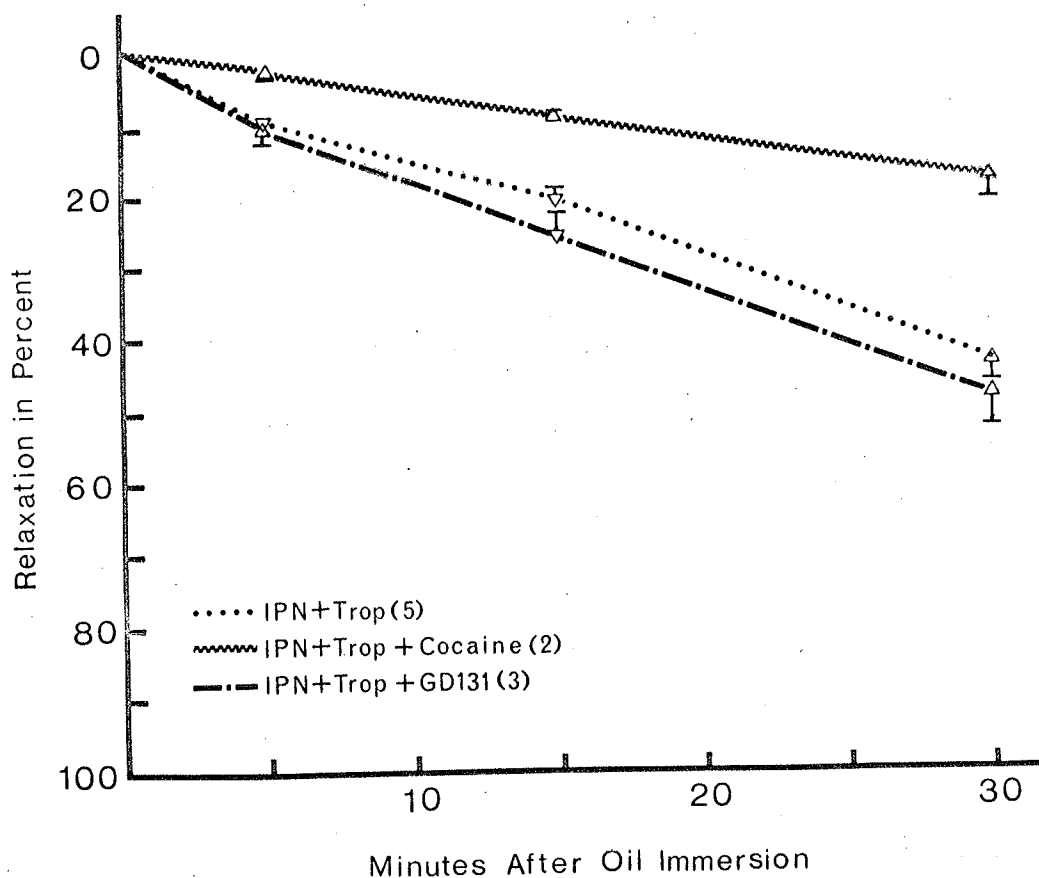
Fig. 52. Effects of GD-131 and Cocaine on the Relaxation in Oil of Noradrenaline Contracted Aortic Strips after Enzyme Inhibition.

All strips were pretreated with iproniazid (IPN) and tropolone (Trop) and contracted by noradrenaline (1 X 10^{-8}). Cocaine (1 X 10^{-4}) and GD-131 (1 X 10^{-5}) were added 10 minutes before oil immersion. Figures in parentehses indicate number of preparations represented by each curve. Bars indicate standard errors of means. Records from 2 of these experiments are shown in figure 51.

Statistical analysis:

	Min.
IPN + Tropolone vs. Iproniazid + Tropolone + GD-131	N.S.
IPN + Tropolone vs. Iproniazid + Tropolone + Cocaine	5 - $P < 0.01$ 15 - $P < 0.01$ 30 - $P < 0.01$

of cocaine. One of 6 strips from each of 4 aortas from reserpinized rabbits was assigned to each of the following treatment groups.

#1. Exposure to GD-131 (1 X 10^{-5}) for 10 minutes, followed by a 30 minute period with frequent washes, contraction by noradrenaline (1 X 10^{-8}) and oil immersion.

#2. Contraction by noradrenaline (1 X 10^{-8}), followed by oil immersion.

#3. Contraction by noradrenaline (1 X 10^{-8}), followed by exposure to cocaine (1 X 10^{-4}) for 10 minutes before oil immersion.

#4. Pretreatment with GD-131 (as in group #1), followed by contraction by noradrenaline (1 X 10^{-8}), cocaine (1 X 10^{-4}) and oil immersion.

#5. Pretreatment with cocaine (1 X 10^{-4}) for 20 minutes, followed without washout by exposure to GD-131 for 10 minutes and subsequent treatment as for group #1.

#6. Pretreatment with cocaine (1 X 10^{-4}) for 20 minutes, followed without washout by exposure to GD-131 for 10 minutes and subsequent treatment as in group #4.

The responses of strips in groups #1 to #4 were described above. The strips of group #5 relaxed in oil at a rate not significantly different from that of group #1 strips. The mean relaxations of strips protected with cocaine (1 X 10^{-4}) (group #5) and of unprotected strips (group #1) were 17.1 and 10.8% and 50.9 and 44.1%, respectively, after 5 and 15 minutes of oil immersion. Responses of strips previously protected by cocaine (group #6) were potentiated 5.0 mm (10.9%) by cocaine (1 X 10^{-4}) and the unprotected strips (group #4) 4.0 mm (7.9%).

These differences were not statistically significant. The cocaine

protected strips of group #6 and the unprotected strips of group #4

relaxed 2.5 and 4.3% and 14.9 and 10.0%, respectively, during 5 and

30 minutes of oil immersion. These differences were also not

statistically significant. These experiments failed to demonstrate

the involvement of common receptor sites for GD-131 and cocaine in

either the potentiation of responses to noradrenaline or in the

inhibition of its inactivation. However, the existence of such sites,

particular in relation to potentiation where the two drugs have a

considerable pharmacological overlap, cannot be ruled out until much

more detailed studies have been performed with a wide range of exposure

times and drug concentrations.

C. DISCUSSION - MECHANISMS OF ACTION OF COCAINE AND GD-131

The observation that cocaine concomitantly depresses the

ability of sympathetically innervated organs to accumulate circulating

catecholamine and potentiates their responses to the amine has led to

formation of a unitary hypothesis of the mechanism of action of cocaine

linking these two effects. This postulates that cocaine blocks a

specialized nerve membrane transport system for sympathomimetic amines

and, thus, their uptake and subsequent intraneuronal metabolism or de-

position in storage granules. This block is assumed to result in the

diversion of increased amounts of amine to the vicinity of the adrener-

gic receptors, with the production of an enhanced response (MacMillan,

1959; Muscholl, 1961; Furchgott et al., 1963; Kopin, 1964; Axelrod,

1965; Hertting, 1965; Trendelenburg, 1965; Carlsson and Waldeck, 1965).

Evidence was presented in the Historical Introduction that sympatheti-
cally innervated tissues can take up circulating amine, and that cocaine
can block this uptake, at least partially (Whitby et al., 1960; Hertting
et al., 1961b; Muscholl, 1961; Kirpekar and Cervoni, 1963; Van Zwieten,
et al., 1965). However, there are certain published observations which
are not entirely consistent with this unitary hypothesis. For example,
it has been reported that pretreatment of rats with cocaine does not
decrease the uptake of circulating catecholamine by the uterus (Wurtman
et al., 1964), although Varagic (1956) reported that cocaine potentiates
responses to both nerve stimulation and exogenous noradrenaline in the
rabbit hypogastric nerve-uterus preparation in vitro. However, it is
clear that these discrepancies have not prevented general acceptance of
the hypothesis.

Several types of experiments described in this thesis are in-
compatible with the unitary hypothesis attributing potentiation of re-
sponses to sympathomimetic amines by cocaine to inhibition of their in-
activation by transport into neurones. The experiments performed with
methoxamine are the most obvious. Methoxamine is not a substrate for
either of the enzymes which inactivate sympathomimetic amines in tissues
(MAO and COMT), and there appear to be no other pathways for its endoge-
nous inactivation in aortic strips. This was confirmed by the observation
that there was no relaxation of reserpinized aortic strips contracted by
methoxamine during an observation period of almost one hour in oil.
After washout of methoxamine (1×10^{-7}) in Krebs solution, relaxation was
59.5% complete in 15 minutes. Despite the complete inability of reser-
pinized aortic strips to inactivate this agonist, cocaine markedly

potentiated their responses to methoxamine, an effect considerably grea-
ter than that which would have been produced by doubling the concentra-
tion of agonist. It should also be noted that the increments in height
of contraction produced by cocaine occurred much more rapidly than did
the initial contraction produced by methoxamine. It would be expected
that the cocaine increments, if due to a diversion of methoxamine to-
wards the receptors, would occur with a similarly gradual slope. These
observations on the interaction of methoxamine and cocaine indicate
that the action of cocaine which potentiates contractile responses to
sympathomimetic amines and that which results in an inhibition of amine
inactivation can be completely dissociated, and that at least a major
part of its potentiating action is unrelated to any effect on inacti-
vation mechanisms.

Other experiments reported in this thesis provide additional
evidence that potentiation of responses to various amines by cocaine
can occur without a concomitant equivalent effect on inactivation. The
results presented in Table XV did not provide a qualitative dissocia-
tion of the potentiation of responses to noradrenaline, adrenaline or
phenylephrine by cocaine from impairment of their inactivation in
either unreserpinized or reserpinized aortic strips, e.g., cocaine
(1×10^{-4}) potentiated responses of reserpinized aortic strips to nor-
adrenaline more than did a concentration of 1×10^{-5}, and also de-
creased their rate of relaxation after oil immersion significantly more.
However, it was found that the percent inhibition of amine inactivation,
calculated from the magnitude of the potentiation, was consistently
greater than that actually measured by inhibition of the rate of re-
laxation of the same strips in oil. The opposite deviation would have

been expected if potentiation were causally related to decreased amine

inactivation, because the elimination by oil immersion, of diffusion

into the bathing medium as a mechanism of amine removal should magnify

the contribution of each endogenous mechanism involved in terminating

its action. A more obvious dissociation of the 2 parameters of cocaine

action was the finding that although cocaine (1×10^{-5}) potentiated the

responses of reserpinized strips contracted with noradrenaline after

pretreatment with iproniazid and tropolone, it did not significantly

slow the relaxation of these strips after oil immersion.

Another line of evidence pointing to two mechanisms of action

of cocaine was the demonstration that it effectively potentiated respon-

ses of iproniazid pretreated aortic strips from reserpinized rabbits to

phenylephrine even after exposure to the sympathomimetic amine for 60

minutes. The doses of reserpine used (as high as 5.0 mg/kg, intramus-

cularly, 16 to 24 hours before death) should have been much more than

sufficient to insure blockade of storage of amines in intraneuronal gra-

nules (Stjarne, 1964; Malmfors, 1965), and the treatment with iproniazid

should have insured the absence of intraneuronal deamination. If the

observed potentiation of responses to phenylephrine by cocaine under

these conditions is to be attributed to blockade of nerve membrane trans-

port, one must assume not only that blockade of incoporation into gra-

nules by reserpine does not alter nerve uptake, but that net uptake is

still undiminished by outward diffusion after a 60 minute exposure to

phenylephrine under conditions which prevent all metabolism of the ac-

cumulated amine.

Further evidence that potentiation of responses to various

amines by cocaine cannot be adequately explained as a result of the blo-

ckade of nerve membrane transport, was the occasional potentiation of

responses to histamine and 5-hydroxytryptamine by cocaine and by methy-

lphenidate, which is believed to have a similar mechanism of action, in

both reserpinized and unreserpinized aortic strips. These amines appear

not to be taken up and stored in adrenergic nerves. Axelrod and Insoe

(1963) found that serotonin is not localized in the same subcellular

fractions of heart as is noradrenaline, and tyramine is incapable of

liberating serotonin from tissues. In addition, fluorescence microsco-

pic studies indicate that adrenergic nerve terminals have a negligible

capacity to take up circulating serotonin (Malmfors, 1965). Similarly,

the observation, reported above, that neither cocaine nor methylpheni-

date delays the inactivation of histamine indicates that nerve uptake

makes no significant contribution to its disposition.

There is also evidence, both from the literature and from ex-

periments reported in this thesis, that drugs can block the disposition

of amines by binding and storage without concomitantly potentiating re-

sponses to them. Because they had long been known to potentiate respon-

ses of certain organs to noradrenaline and adrenaline, Isaac and Goth

(1965) compared the ability of a group of antihistamines to potentiate

the chronotropic action of noradrenaline on spontaneously beating rat

atria in vitro with their effects on the uptake of noradrenaline by the

rat heart. The latter was determined by pretreating rats with an anti-

histamine and removing and assaying their hearts for radioactivity 30

minutes after the administration of H^3-noradrenaline. Although there

was a correlation between the two effects of many antihistamines, one of

the most effective inhibitors of H^3-noradrenaline uptake (phenindamine)

produced no potentiation. This finding was termed "paradoxical" by the

authors.

Maxwell (1965 a,b) found that both guanethidine and methylphenidate impaired the uptake and binding of H^3-noradrenaline by aortic strips and potentiated their responses to catecholamines, but the two effects were not well correlated, e.g., maximal inhibition of noradrenaline uptake and binding was produced by lower concentrations of methylphenidate than was maximal potentiation. Maxwell suggested that these compounds potentiated responses to noradrenaline by altering its interaction with adrenergic receptors, thus, producing a state of hyperresponsivity.

The results of the present experiments with procaine demonstrated quite clearly that potentiation can be produced without impairment of amine inactivation and vice versa. It was found that procaine inhibited the relaxation of phenylephrine contracted strips in oil, apparently by the same mechanism as cocaine, in that both eliminated the residual capacity of iproniazid pretreated aortic strips to inactivate phenylephrine. However, procaine did not potentiate responses to phenylephrine in any experiment. Although it did not appear to have any important effect on inactivation other than that shared with procaine, cocaine administered in the presence of procaine effectively potentiated responses to both phenylephrine and noradrenaline. Thus, this one series of experiments provided examples of both inhibition of amine inactivation without potentiation and potentiation without concomitant depression of an inactivation mechanism.

Inhibition of an inactivation mechanism by procaine should increase the biophase concentration of agonist under the steady state

conditions which probably exist after a drug induced contraction of an

aortic strip has reached a plateau height and, thus, should potentiate

the response. That such an effect was not seen indicates the lack of

importance of the procaine sensitive mechanism (apparently equivalent to

the cocaine sensitive mechanism) in amine inactivation in the face of

other pathways, including diffusion into the bathing medium. It is pos-

sible that under other conditions this inhibition of amine inactivation

could lead to some potentiation of responses to sympathomimetic amines,

which could account for the conflicting reports that procaine does (Bacq

and Lefebvre, 1935; Armin et al., 1953) and does not (Tainter, 1930;

Wirt and Tainter, 1932) potentiate.

Since the results discussed above indicated that potentiation

of responses to sympathomimetic amines by cocaine is independent of its

effect on their inactivation, the locus of this potentiating action was

investigated in experiments designed to distinguish between nerve cell

and effector cell loci. Cocaine effectively potentiated responses to

noradrenaline in strips which had been stored at $6^{o}C$ for as long as 10

days, although the characteristic response to tyramine was absent after

4 days of storage (the earliest tyramine test). Similarly, cocaine po-

tentiated responses of strips kept in the muscle chambers at $37^{o}C$ for as

long as 28 hours, when the initial response to noradrenaline had already

markedly deteriorated. These results provide strong evidence that po-

tentiation by cocaine is due to an action on effector cells rather than

on nervous elements, because the latter must have become nonfunctional

long before the latest demonstrations of cocaine potentiation in both

series.

Malmfors and Sachs (1965) studied the effects of superior cervical ganglionectomy on adrenergic nerves in the rat iris by the technique of fluorescence microscopy and found that a "large portion of the terminals were affected and lost their noradrenaline during the four hours between 12 and 16 hours after axotomy". Most of the terminals had "disappeared" in 16 to 24 hours and no accumulation of exogenous noradrenaline or other amines could be demonstrated 24 hours after axotomy. The close association between loss of stored transmitter and loss of "uptake" capacity was taken to "strongly support the view that these mechanisms in any given system of terminals operated up to a certain time without any obvious changes, but then deteriorated rapidly and at about the same time as the transmitter stores disappeared". Far distal axotomy by removal of the aorta should lead to the degenration of nerve terminals even more rapidly than that observed in the experiments of Malmfors and Sachs. Denervation supersensitivity develops more rapidly the shorter the distal segment of a sectioned cholinergic nerve (Emmelin and Malm, 1965), probably because materials transported along the axon and essential for the synthesis of mediator are available longer when the "distal stump of the severed nerve is long than when it is short". Similar transport is probably essential for the integrity of adrenergic nerve terminals (Dahlstrom, et al., 1965.)

Dibenamine and phenoxybenzamine have been reported by a number of investigators to inhibit the inactivation of noradrenaline (Brown and Gillespie, 1957; Bacq et al., 1960; Kirpekar and Cervoni, 1963; Blakeley et al., 1963; Gillespie and Kirpekar, 1965), presumably because of the blockade of α adrenergic receptors. In the experiments reported

in this section another β-haloalkylamine, GD-131, was found both to po-
tentiate contractions of aortic strips induced by noradrenaline and to
impair the inactivation of this amine in concentrations which produced
no detectable α adrenergic blockade. These findings make it unnecessary
to implicate the adrenergic receptors in the termination of action of
noradrenaline or in the alteration of amine disposal caused by Dibena-
mine or phenoxybenzamine.

Interpretation of the effects of GD-131 in terms of mechan-
isms for the disposition of sympathomimetic amines requires careful
assessment of: 1) The nature and magnitude of the potentiation of re-
sponses to noradrenaline by GD-131, and its relationship to the similar
effect of cocaine, and 2) the nature and magnitude of the interference
with noradrenaline inactivation by GD-131, and the relationship of this
action to that of cocaine.

The present results can be best interpreted as demonstrating
a partial overlap of the mechanisms through which GD-131 and cocaine po-
tentiate responses to noradrenaline. It appears that most, but not all,
of the effect produced by cocaine can be duplicated by GD-131, which
has, in addition, a fairly large component of action not shared by co-
caine. GD-131 potentiated responses to noradrenaline significantly
more than the maximal potentiation by cocaine. In one specific compari-
son, cocaine (1×10^{-4}) and GD-131 (1×10^{-5}) increased the amplitude
of response to noradrenaline (1×10^{-8}) by 28.2 and 37.1%, respectively
equivalent to increasing the concentration of noradrenaline to 4×10^{-8}
and 7×10^{-8} (Table XIXa). In addition, cocaine produced only a meagre
effect when added after treatment with GD-131, whereas the latter still

produced a potentiation equivalent to doubling the noradrenaline concentration when given in the presence of a maximally potentiating concentration of cocaine.

Furchgott (1960c) found that Dibenamine potentiated the inhibitory responses of rabbit intestinal strips to isoproterenol, and Stafford (1963) reported that cocaine does not potentiate responses of the rabbit intestine to catecholamines. Taken together, these reports suggest that potentiation by β-haloalkylamines and by cocaine might involve different mechanisms. The present experiments showed one clear difference between potentiation by cocaine and by GD-131 in that the former reliably depressed contractions of aortic strips induced by tyramine, presumably by blocking its transport to intraneuronal sites of catecholamine stores, whereas GD-131 consistently potentiated them. Conversely, a component of similarity or identity in the actions of these two drugs was shown by the fact that both potentiated contractile responses to methoxamine to a similar degree. This common component of action must be directly on effector cells because it occurred in the absence of any demonstrable inactivation of methoxamine by the aortic strips which could provide a basis for an indirect action.

Both cocaine and GD-131 have parameters of action which reduce the rate of inactivation of noradrenaline in aortic strips. Treatment of aortic strips with GD-131 (1×10^{-5}) for 10 minutes or with cocaine inhibited noradrenaline inactivation about equally (Fig. 46). The shifts in times for half relaxation produced by either cocaine or GD-131 alone indicated blockade of about 50% of the inactivating capacity of the strips. This might suggest actions on the same inactivation

pathway. However, the effects were roughly additive, both in experiments in which cocaine was added to the chambers of strips contracted by noradrenaline after pretreatment with GD-131, and in experiments in which both drugs were added to the chambers after noradrenaline contractions had reached a plateau.

It appears that although some overlap in the actions of cocaine and GD-131 which impair the inactivation of noradrenaline by aortic tissue cannot be ruled out; this was clearly not great under the conditions of the above experiments. In contrast, the combination of methylphenidate (1×10^{-6}) and cocaine (1×10^{-5}) inhibited the inactivation of phenylephrine no more than did either alone, as would be expected of two agents with a common mechanism of action.

The ability of aortic strips to inactivate noradrenaline could be almost eliminated by exposure for 30 minutes to GD-131 (1×10^{-5}). Three strips so treated and then contracted by noradrenaline (1×10^{-8}) relaxed a mean of 33.2% during 30 minutes in oil, one only 18.9%. The rate of relaxation of this strip was similar to that of strips treated with the combination of iproniazid, tropolone and cocaine (1×10^{-4}). Pretreatment for longer periods with this concentration of GD-131 produced a gradually increasing α adrenergic receptor blockade, which prevented reliable testing.

Study of the effects of cocaine in the presence of GD-131 showed inhibition of the inactivation of noradrenaline and potentiation to be independent. Cocaine (1×10^{-4}) exerted its typical effect on rates of relaxation in aortic strips pretreated with GD-131, but produced only slight additional potentiation of responses to noradrenaline

(Table XIX).

The effect of cocaine on inactivation of noradrenaline was found to be approximately additive with that of enzyme inhibition, and since the effects of GD-131 and cocaine were largely additive, i.e., appeared to involve different mechanisms, the relationship between the GD-131 mechanism and enzymatic inactivation of noradrenaline was explored. Exposure of strips to GD-131 (1×10^{-5}) 10 to 15 minutes before oil immersion inhibited the inactivation of noradrenaline more than did inhibition of COMT. However, after inhibition of both MAO and COMT, a 10 to 15 minute exposure to GD-131 (1×10^{-5}) produced no further inhibition of inactivation, whereas cocaine was still effective. Thus, this slightly submaximal treatment with GD-131 appears not to block the major mechanism through which cocaine inhibits the inactivation of noradrenaline, presumably the transport of amine across cell membranes to sites of binding and storage. A similar differentiation was demonstrated in the experiments on potentiation of responses to tyramine, which showed clearly that GD-131 had not blocked the transport of tyramine to sites of storage of endogenous catecholamines.

The β-haloalkylamines have been reported not to inhibit COMT (Axelrod, 1960b), and there is no evidence that they can inhibit MAO under the conditions of the present experiments. However, moderate exposure to GD-131 (1×10^{-5} for 10 to 15 min.) appeared to produce essentially the same effect as inhibition of both enzymes; the inhibitions of relaxation were nearly equivalent and GD-131 had little effect on strips in which both enzymes had been inhibited. In addition, both were additive with the major effect of cocaine. More prolonged incubation with GD-131 appeared to be capable of inhibiting noradrenaline in-

activation as much as the combined effects of iproniazid, tropolone and cocaine, although this was difficult to demonstrate consistently, probably because the exposure required was very close to that which produced significant α adrenergic blockade. These observations suggested that GD-131 might inhibit all endogenous routes of noradrenaline inactivation by a single basic mechanism of action, to which the cocaine sensitive pathway is quantitatively more resistant.

The hypothesis is proposed here that the effects of GD-131 on amine inactivation are due to a primary action which inhibits the movement of noradrenaline and other sympathomimetic amines through membranes. Under the conditions of the present experiments this action involved no fundamental alteration of the contractile machinery or generalized impairment of the movement of all drugs across membranes. This was demonstrated by the failure of GD-131 to inhibit the inactivation of histamine or of a concentration 10 times higher than that usually employed to slow the relaxation of strips contracted by angiotensin.

A model which could explain the observed effects of GD-131 and cocaine on the magnitude of responses to noradrenaline and on its rate of inactivation is presented in figure 53a. Impairment of noradrenaline inactivation by exposure of aortic strips to GD-131 (1×10^{-5} for 10 to 15 min.) appears to result from extensive blockade of the access of noradrenaline to sites of degradation in effector (smooth muscle) cells. This effect is approximately equal to and not additive with that of inhibiting both MAO and COMT. Longer incubation with GD-131 results in completion of this blockade, and a partial blockade of the transport at both nerve and effector cell membranes which leads to

binding and storage of amine.

The second action of GD-131 mentioned is the same as the primary action of cocaine, i.e., cocaine inhibits the inactivation of sympathomimetic amines by blocking the transport mechanisms of both nerve and effector cell membranes which lead to their uptake and storage. Cocaine also can inhibit the access of these amines to sites of enzymatic degradation, although the magnitude of this effect was not great under the conditions of the present experiments. (See also Section V, H and General Discussion.)

In summary, the primary action of GD-131 affecting the disposition of noradrenaline and of other sympathomimetic amines is to impair their movement through membranes. This is first manifest as inhibition of access to sites of enzymatic inactivation, but with increased exposure, the transport of amines through nerve and effector cell membranes to sites of storage and binding is also impaired. Cocaine also inhibits the movement of amines across membranes, but its predominant action is to block transport to sites of storage in both nerves and effector cells, access to sites of metabolism is inhibited considerably less effectively.

The potentiation of responses to sympathomimetic amines produced by cocaine can be attributed predominantly to some action on effector cells which makes them hyperresponsive (Fig. 53b) and which is entirely independent of any alteration in their rates of inactivation. Under some conditions a small component of the potentiation produced by cocaine may be secondary to impairment of the inactivation of amines by storage and binding and by enzymatic inactivation.

GD-131 shares with cocaine the action on effector cells which makes them hyperresponsive to certain amines (Fig. 53b) The potentiation of responses to sympathomimetic amines by GD-131 appears not to involve blockade of transport to sites of storage and binding except possibly after maximal exposure short of α adrenergic blockade. However, an action which simulates enzyme inhibition may contribute to potentiation by GD-131. This effect on amine disposition appears to be due to blockade of their access to the sites of enzymatic inactivation by MAO and COMT, predominantly in effector cells. This action of GD-131 may effectively decrease the volume of distribution of noradrenaline and other amines within the cells, but this can only be conjectural until the location of the barriers involved is known.

The evidence adduced to support various aspects of the hypothesis depicted in figure 53 can be summarized as follows:

a)   Evidence that potentiation of responses to sympathomimetic amines by cocaine and GD-131 is largely independent of blockade of nerve membrane transport and other mechanisms for their inactivation.

1)   Potentiation by cocaine was unaltered after a 60 minute exposure of reserpinized, iproniazid pretreated aortic strips to phenylephrine. In the absence of both intraneuronal storage and metabolism, net uptake of amine by nerves should have been markedly reduced, with a concomitant reduction in the potentiation produced by cocaine if this were a result of blockade of nerve membrane transport of amine.

2)   Potentiation by cocaine and methylphenidate of some responses to amines (histamine and 5-hydroxytryptamine) which appear not to be taken up and stored in adrenergic nerves.

3)   Cocaine still effectively potentiated responses of aortic

strips to noradrenaline after their neuronal elements had been allowed
to degenerate during prolonged periods in the cold and at 37°C.

4)   Both cocaine and GD-131 effectively potentiated responses of
aortic strips to methoxamine, although studies by the oil immersion
technique confirmed the complete absence of endogenous mechanisms for
the inactivation of this sympathomimetic.

5)   Both procaine and cocaine virtually eliminated the residual
inactivation of phenylephrine in iproniazid pretreated strips, but only
the latter potentiated responses to this amine. Cocaine still effectiv-
ely potentiated responses to phenylephrine in the presence of procaine.

6)   The increased concentrations of active amines in the vicinity
of tissue receptors which could result from the delay in inactivation
produced by cocaine or GD-131 were inadequate to account for and were
poorly correlated with the potentiation produced by these agents.

b)   Evidence that a major part of the potentiation produced by cocaine
      and by GD-131 involves a common mechanism, but that a part of the
      effect of each is on independent mechanisms, related to their ef-
      fects on mechanisms of inactivation.

(1   Cocaine and GD-131 produced almost identical potentiation of
responses to methoxamine, an effect entirely unrelated to inactivation
of the amine.

2) Cocaine produced a minor, but reproducible, potentiation of res-
ponses to noradrenaline in the presence of GD-131.

3)   GD-131 caused a somewhat reduced, but still considerable, po-
tentiation of responses to noradrenaline in the presence of cocaine.

4)   Inhibition of COMT markedly decreased the potentiation of res-
ponses to tyramine in iproniazid pretreated aortic strips by GD-131, and

and may have decreased that in otherwise untreated noradrenaline contracted strips. Inhibition of COMT also somewhat decreased the potentiation produced by cocaine (1×10^{-4}).

c)   Evidence that the major effects of GD-131 and cocaine on the inactivation of noradrenaline are independent.

1)   Impairment of the inactivation of noradrenaline by moderate exposures to GD-131 (1×10^{-5} for 10 to 15 min.) and by cocaine (1×10^{-4}) was approximately additive.

2)   Moderate exposure of aortic strips to GD-131 reduced the rate of inactivation of noradrenaline more than did inhibition of either MAO or COMT.

3)   Cocaine produced a further reduction in the rate of inactivation of noradrenaline by aortic strips in which both COMT and MAO had been inhibited, but moderate exposure to GD-131 did not, indicating that the major action of the latter is on access of the amine to sites of enzymatic degradation and not on transport to sites of binding and storage.

4)   GD-131 potentiated responses to tyramine, whereas cocaine inhibited them. This provided further evidence that the major action of GD-131 is not on nerve membrane transport of amine to storage sites.

5)   Maximal exposure to GD-131 short of producing α adrenergic blockade sometimes inhibited the inactivation of noradrenaline as much as combined treatment with iproniazid, tropolone and cocaine, which indicates that this compound has also the ability to block the binding and storage of amines, as does cocaine.

6)   Cocaine appeared to have minor components of action comparable to the major properties of GD-131. It produced a smaller decrease in

the inactivation of phenylephrine and adrenaline in aortic strips in which MAO and COMT, respectively, had been inhibited than in the controls, indicating some overlap with the major pathway of amine metabolism in each case. Cocaine also appeared capable of blocking movement of amine to extraneuronal as well as intraneuronal binding and storage sites. (See General Discussion.)

d)   Evidence that the mechanisms of inactivation inhibited by cocaine and GD-131 are specific for sympathomimetic amines.

1) Neither cocaine or methylphenidate delayed the inactivation of histamine.

2) GD-131 did not delay the inactivation of either histamine or angiotensin.

It is possible to reconcile the above findings and the proposed theory of the mechanisms of action of cocaine with most of the pertinent published observations. The blockade of amine uptake by nerves, which is an unquestioned parameter of the action of cocaine, undoubtedly can result in an increased concentration of noradrenaline in the environment of the tissue receptors, but even if half of the catecholamine which might reach the receptors were extracted by ramifications of the sympathetic plexus in densely innervated organs such as the nictitating membrane and heart, the maximal effect would be a very slight potentiation, equivalent to that of doubling the concentration of amine. The potentiation of responses of the nictitating membrane and heart to noradrenaline produced by cocaine has been reported to be equivalent to at least 30 and 10 fold increases in amine concentration, respectively (Haefely et al., 1964; Trendelenburg, 1965; Furchgott et al., 1963). Thus, some other mechanism, presumably the direct action on ef-

fector cells demonstrated in the experiments described above, is necessary to account for the magnitude of the potentiation produced by this agent. It appears that recognition of the existence of such a mechanism has been prevented by the general acceptance of the unitary hypotheses of cocaine action.

The observation that cocaine does not potentiate the responses of denervated organs to catecholamines has been one of the cornerstones of the hypothesis which attributes potentiation by this agent to blockade of the transport of amines into nerve cells. However, this finding may be interpreted with equal justification as indicating that the changes in effector cells following denervation are similar to those produced by cocaine. There is convincing evidence that denervation supersensitivity cannot be adequately explained by the absence of nerve uptake sites. (See review by Emmelin, 1961.) However, this has been largely ignored in the use of observations on denervated organs to support the unitary hypothesis of cocaine action.

The following General Discussion will attempt to integrate the observations presented in this thesis and to use them as a basis for examining mechanisms which may be involved in terminating the action of the sympathetic mediator.

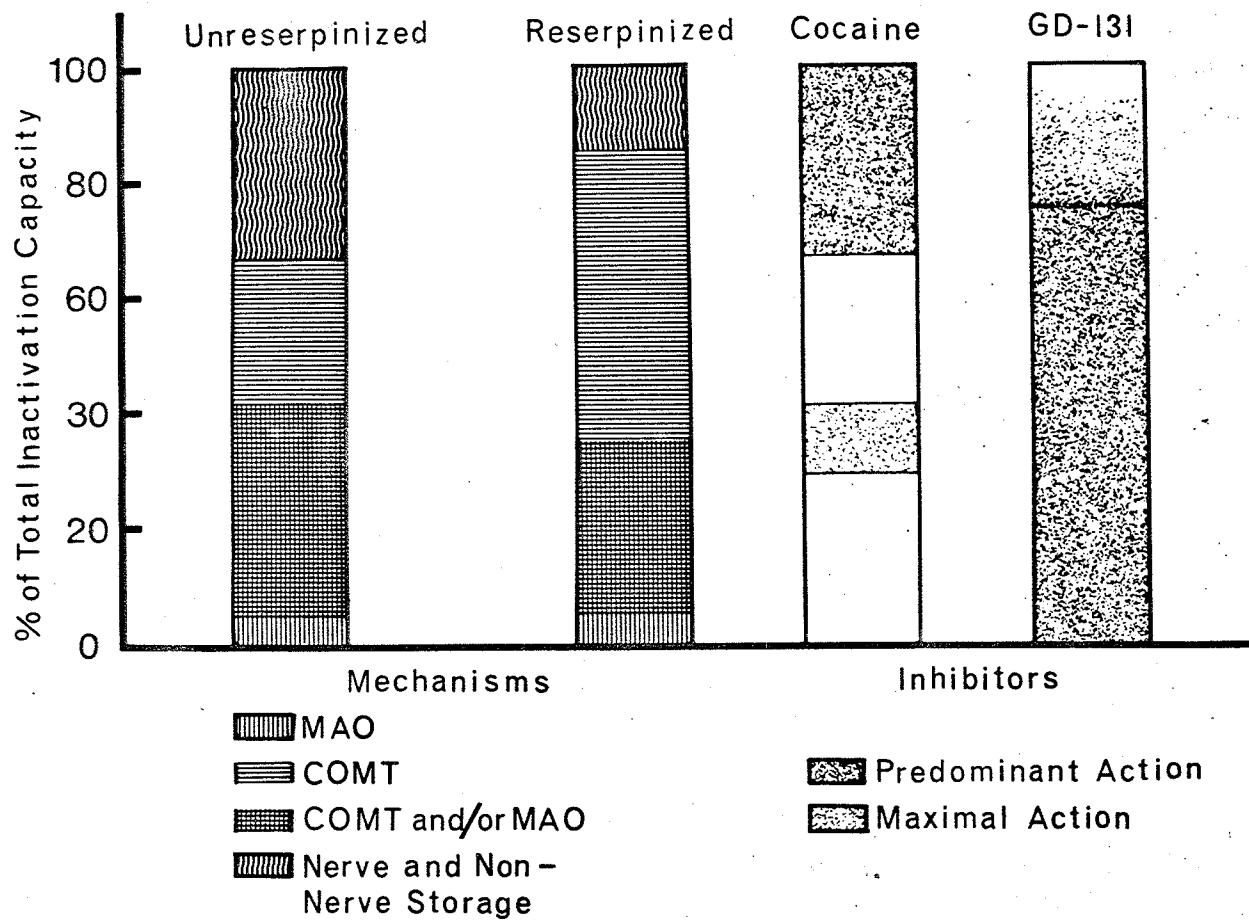Fig. 53a. Mechanisms for the Endogenous Inactivation of Nor-

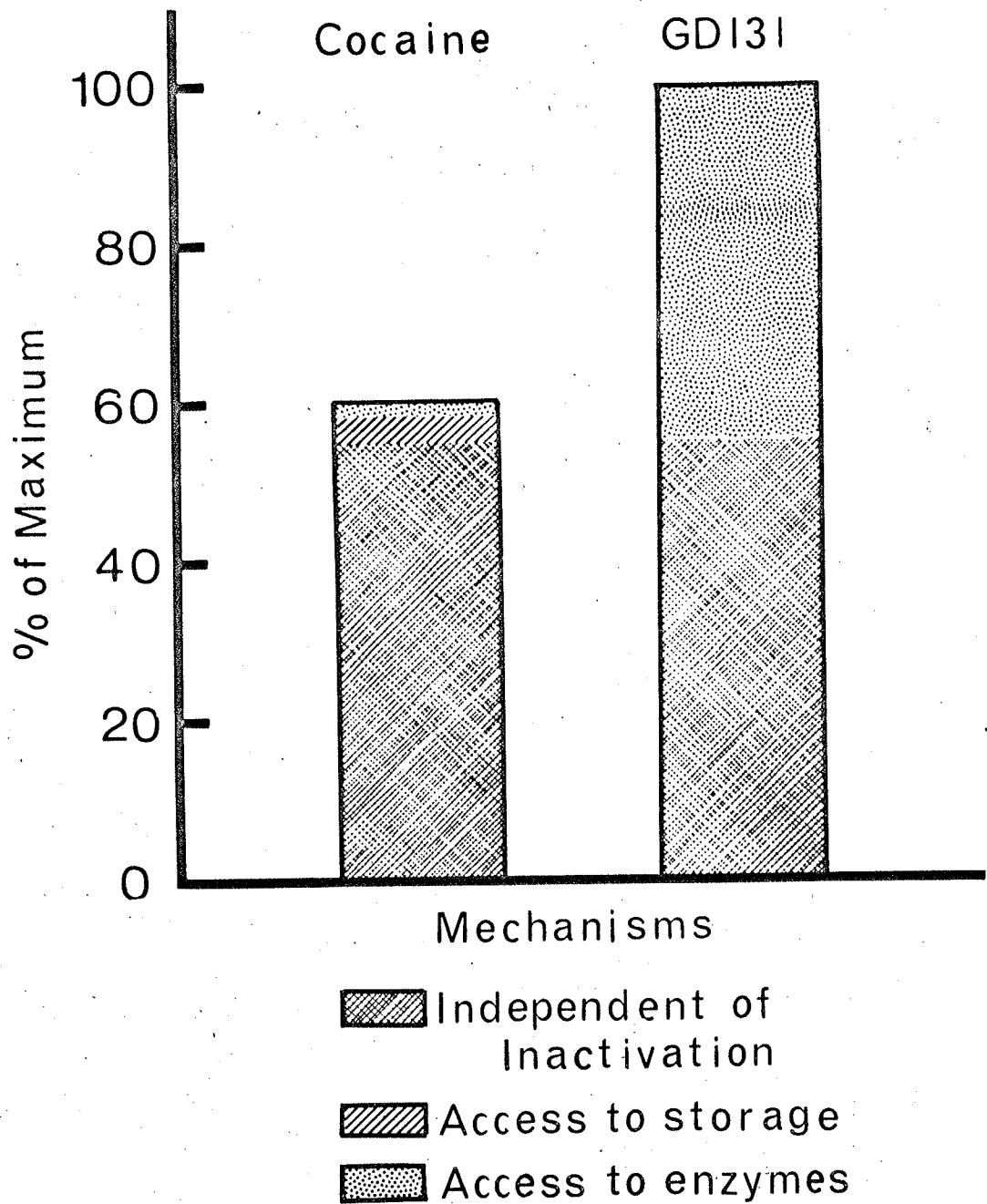adrenaline in Relation to the Actions of Cocaine

and GD-131.

Fig. 53b. Mechanisms by which Responses to Noradrenaline
are Potentiated by Cocaine and GD-131.

The maximal potentiation produced by GD-131
has been arbitrarily assigned the value of 100%.

VIII. GENERAL DISCUSSION - A THEORY TO EXPLAIN THE TERMINATION OF ACTION OF THE SYMPATHETIC MEDIATOR

Termination of the action of noradrenaline released by adren-
ergic nerve activity is currently believed by a large majority of workers
in the field to be due to uptake of amine by sympathetic nerves, involv-
ing an active membrane transport process. Two major lines of evidence
are used to support this hypothesis:

(a) Sympathetically innervated organs can accumulate exogenous
catecholamine, and uptake, but not net accumulation, has been demonstra-
ted with concentrations of circulating tritiated amine believed to be
no greater than those which occur physiologically.

(b) Pretreatment with cocaine results in an increase in efflu-
ent noradrenaline following sympathetic nerve stimulation, and potentia-
tion of organ responses.

Although acknowledging the existence of a nerve membrane trans-
port mechanism capable of extracting circulating noradrenaline with high
efficiency, Strömblad and Nickerson (1961) and Nickerson (1965) ques-
tioned the validity of assuming that the appearance of tritiated amine
in tissues, represents accumulation (net uptake) rather than exchange.
Both processes are known to occur at sites of amine storage, and differ-
entiation is crucial if the role of transport of amine into nerves in
the termination of the action of noradrenaline is to be assessed.

Crout (1964) attempted to resolve this point by the use of re-
latively high doses of tritiated amine so that the concentrations of
tritiated, endogenous and total noradrenaline could be determined in the
guinea pig heart at various times after the administration of label.
One minute after the intravenous injection of H^3-noradrenaline (3.5
umol/kg) the total noradrenaline concentration of the heart was almost

doubled. During the first 15 minutes after injection about 45% of the H^3-noradrenaline taken up by the heart was lost and loss continued thereafter at a markedly reduced rate. Total noradrenaline content decreased progressively and reached the control level about 4 hours after the injection. Endogenous noradrenaline was apparently unchanged one hour after the administration of H^3-noradrenaline, but dropped below normal at the next determination, which was 4 hours after administration of the label. Crout concluded that exogenous noradrenaline can slowly displace endogenous amine from stores, but this takes place slowly over a period of hours, and the nerve membrane transport mechanism can accomplish an earlier net uptake of noradrenaline.

The physiological implications of these experiments are limited because of the very large doses of amine administered. However, they suggest that although the appearance of tritiated amine in tissues cannot always be translated with assurance into "net uptake", amine extracted from the bloodstream can be added to endogenous stores for a sufficient period of time to permit this mechanism to function in terminating the action of amine released by adrenergic nerves.

Assuming that cocaine potentiates responses to catecholamines by blocking nerve uptake and, thus, increasing the amount available to activate tissue receptors, Haefely et al. (1964) found potentiation of nictitating membrane responses to postganglionic nerve stimulation by this agent to be of a magnitude such as to suggest that less than 5% of the total mediator released normally reaches the vicinity of the receptors. The potentiation of responses to sympathomimetic amines by cocaine, and its assumed mechanism of action, have been used by many other workers

as a cornerstone for both qualitative and quantitative conclusions re-garding the mechanisms terminating the action of noradrenaline. However, experiments reported in this thesis have shown that the major component of this potentiation is unrelated to processes of amine disposition. Consequently, it cannot be used, either quantitatively or qualitatively, as an indicator of amine normally returned to storage in nerves.

Direct analysis of the amount of noradrenaline in the effluent from control and cocaine pretreated organs during nerve stimulation pro-vides a somewhat better index of amine returned to storage after sympath-etic nerve stimulation, and on the basis of published figures from such studies it appears that restorage may account for less than 50% of the total noradrenaline released. Marked potentiation could result from blockade of nerve uptake only if most of the amine released is returned to storage before it reaches the adrenergic receptors. Such amine would not contribute to body function or economy, except in the sense that a large release with uptake would be equivalent to a smaller release with-out it. The crucial mechanisms which terminate, and therefore regulate, actions of the sympathetic mediator must deal with amine which reaches tissue receptors.

Experiments reported in this thesis have provided information on the following points, which are pertinent to a discussion of the mech-anism of termination of the action of sympathetic mediator.

a) A major part of low concentrations of both noradrenaline and adrenaline can penetrate effector cells to be enzymatically degraded.

b) Metabolic inactivation can effectively compete with nerve uptake and storage as a means of terminating the action of noradrenaline

and adrenaline.

c) Potentiation of responses to sympathomimetic amines by co-caine is predominantly due to an increased responsiveness of effector cells, unrelated to any effect of this drug on mechanisms of amine dis-position.

d) Cocaine appears to impair the inactivation of sympathomimetic amines by an action on effector cells as well as on adrenergic nerves.

e) Inhibition of amine inactivation by a β-haloalkylamine, commonly seen as an elevated effluent output of noradrenaline after sym-pathetic nerve stimulation, can best be explained as due to a blockade of the access of amines to sites of degradation in effector cells. It may be associated with a decreased volume of distribution of these amines within effector cells.

On the basis of the observations presented, it is postulated that a major portion of the mediator released by physiologically occur-ring sympathetic nerve activity participates in the activation of tissue receptors and that its actions are terminated by movement away from the region of the receptors. A portion of the mediator moves through the interstitial fluid and is removed by the circulation, a portion diffuses to the neuronal ground plexus and is taken up by adrenergic nerves, but the largest portion passes into cells other than nerves and is distribu-ted in cell water. It is this movement which terminates receptor activa-tion. The eventual fate of this amine is enzymatic inactivation (deamin-ation, O-methylation, or both) or binding.

An attempt will be made in the present discussion to reconcile this hypothesis with published observations on adrenergic mechanisms and

drugs affecting them, and to relate it to current concepts of adrenergic nerve function. Major questions to be considered include the following, which will be discussed in sequence.

1) Can the sympathetic nerves lose a sizeable portion of the amine which functions as a transmitter in receptor activation after each nerve impulse without impairment of function? 2) Can noradrenaline presented to tissues in physiological concentrations enter the effector cells? 3) What is the basis for the increased effluent output of noradrenaline following sympathetic nerve stimulation in the presence of cocaine or a β-haloalkylamine? 4) Can the observed levels of noradrenaline and its metabolites in venous effluents after sympathetic nerve stimulation be explained by a mechanism of termination of action based on movement into effector cells to ultimate sites of degradation and binding?

1) Malmfors (1964, 1965), using the fluorescence microscopic method of Falck and Hillarp, found that stimulation of the cervical sympathetic trunk for 30 to 60 minutes at a frequency of 20/sec., far above the maximal physiological rate, produced no obvious change in the noradrenaline content of nerves in the rat iris. After treatment with inhibitors of noradrenaline biosynthesis (H 22/54 or H 33/07) "sympathetic stimulation produced a marked to almost complete disappearance of the transmitter", but the changes after imipramine, which prevents the uptake of exogenous noradrenaline by tissues even more effectively than does cocaine, were "much less marked". These workers confirmed that H 22/54 (also an inhibitor of COMT) had no cocaine-like action preventing the uptake of exogenous amine by tissues, but suggested that imipramine might cause some inhibition of catecholamine synthesis.

These results show that synthesis can maintain a near normal
intraneuronal level of noradrenaline without reuptake even during nerve
stimulation at rates far above the physiological maximum, and that the
return to storage in nerves of a large part of the released amine is not
required to maintain adequate intraneuronal stores of transmitter.

2) There is at present considerable evidence that effector
cells have a high capacity to take up noradrenaline. Experiments with
the oil immersion technique described in this thesis clearly demonstrated
termination of the action of low concentrations of noradrenaline and
adrenaline by intracellular enzymes. Observations indicating that a
major part of the total organ content of MAO and, particularly, of COMT
must be in cells other than nerves have already been presented and dis-
cussed (Section V, H).

There is also considerable published evidence that cells other
than adrenergic nerves can take up and retain catecholamines. Wurtman
et al. (1964) showed that long term storage of catecholamines is not
limited to intraneuronal "dense-core" vesicles. They found that although
rat uterus initially bound, per gram of tissue, only about 1/10th as much
H^3-noradrenaline and -adrenaline as did the heart, both organs released
the amine at similar rates over 48 hours. Fractionation of the tissues
revealed that most of the radioactivity in the heart was particle bound,
but even 3 days after administration most of that in the uterus was in
the supernatant fraction. The distribution of endogenous catecholamines
was reported to be basically the same as that of retained label. An
extraneuronal site of the amines retained in soluble form is indicated
by the observation that tyramine taken up by chronically denervated rat

salivary glands is found predominantly in the soluble fraction of tissue homogenates (Musacchio et al., 1965).

Many studies which appear to show very much lower "uptake" of amines in denervated than in innervated organs have not considered the effects of intracellular metabolism, which may make the content of unchanged amine a very poor measure of the amount taken up from extracellular fluid over a given period of time. This is well illustrated by the study of Almgren et al. (1965) on the rat salivary gland uptake of C^{14}-tyramine, which is rapidly converted to octopamine in sympathetic nerves. Five minutes after an intravenous injection of C^{14}-tyramine, innervated and chronically denervated glands contained the same total amount of C^{14}-tyramine plus octopamine. After 30 minutes the total was 6-fold greater in the innervated tissues. However, an almost equivalent total uptake and retention after 30 minutes was demonstrated in experiments performed after inhibition of monoamine oxidase (MAO), 40.6 and 7.0 ng of tyramine, and 3.4 and 60.0 ng of octopamine in denervated and innervated glands, respectively.

After duct ligation and subsequent atrophy of the gland parenchyma, tyramine uptake was markedly impaired, although blood flow was unaltered. These results indicated that although the conversion of tyramine to octopamine is intraneuronal, the bulk of the tyramine is taken up by nonnervous tissue. Extraneuronal retention of tyramine has also been reported by Fischer et al. (1964).

Observations on extraneuronal uptake of noradrenaline are similar to those on tyramine. Strömblad (1959) reported that chronically denervated cat submaxillary glands contained the same total radioactivity

as contralateral innervated glands after the injection of H^3-adrenaline into the lingual artery, but did not report the time between injection of label and removal of the glands. Anden et al. (1963) determined the H^3-noradrenaline and H^3-normetanephrine in innervated and chronically denervated rat submaxillary glands 30 minutes after the intravenous injection of H^3-noradrenaline (1.0 ug/kg). Their figure 1 shows the H^3-noradrenaline content of denervated glands to be only about 10% that of the controls. However, the former contained more H^3-normetanephrine, and there appears to be only a slight difference between innervated and denervated glands in the sum of the two. Denervated glands from animals treated with reserpine and MAO and COMT inhibitors appear to contain as much, if not more, H^3-noradrenaline as the innervated controls. More surprisingly, there is no apparent difference between the content of these glands and that of untreated, innervated control glands. These results indicate considerable extraneuronal uptake and retention of catecholamine, which can easily be masked by the rapid extraneuronal metabolism of noradrenaline. Extraneuronal retention of H^3-noradrenaline in denervated rat salivary glands was also reported by Fischer et al. (1965).

Studies on immunosympathectomized (i.s.) rats have also shown extensive extraneuronal uptake and rapid metabolism of noradrenaline. Fluorescence microscopy has shown a complete absence of neuronal noradrenaline in several organs of i.s. rats, including the submaxillary gland and heart (Hamberger et al., 1965). Sjöqvist et al. (1965) found that 30 minutes after the intravenous injection of H^3-noradrenaline the label in i.s. hearts was about 16% of that in normals, and that i.s. and nor-

mal submaxillary glands differed only slightly in label content. However, noradrenaline accounted for 79% of the label in normal and only 29% in i.s. hearts, and although total tracer content was almost the same as in normal submaxillary glands, i.s. glands showed a similar reduction in noradrenaline and increase in metabolites. A portion of the H^3-noradrenaline in normal, but not in i.s. hearts could be recovered in a granular fraction of the tissue. These experiments provide clear evidence of extensive uptake and rapid metabolism, both deamination and O-methylation, of noradrenaline by organs deprived of sympathetic innervation.

Indirect evidence of extraneuronal uptake and retention of noradrenaline was provided by the finding that responses of chronically denervated nictitating membranes of reserpinized cats to tyramine were partially restored for as long as 20 to 50 minutes after termination of an infusion of noradrenaline (Trendelenburg and Pfeffer, 1964). Release of catecholamines from extraneuronal sources by tyramine has also been reported by Fischer et al. (1965).

The various lines of evidence discussed above (point 2) appear to demonstrate that considerable amounts of noradrenaline and of other sympathomimetic amines can penetrate effector cells to sites of metabolic inactivation. Consequently, the uptake and retention of amines by sympathetically innervated structures cannot be equated directly with activities of nervous structures.

3) The ability of cocaine to impair the uptake of catecholamines by adrenergic nerves is unquestioned, but the magnitude of this effect and its importance in determining the disposition of the amines under various conditions are not clear. The finding that cocaine can

increase the quantity of noradrenaline in the venous effluent of organs

concomitant with nerve stimulation has been key evidence for the hypo-

thesis that nerve uptake is the major mechanism terminating its action.

The most commonly employed preparation with a major smooth muscle com-

ponent is the cat spleen, and only rarely have marked increases in nor-

adrenaline output from this organ been reported (Thoenen et al., 1964).

More often cocaine has been found to produce either a minor or no in-

crease in effluent noradrenaline (Trendelenburg, 1959; Blakeley et al.,

1963; Kirpekar and Cervoni, 1963).

As discussed above, an elevation in the concentration of efflu-

ent noradrenaline after sympathetic nerve stimulation produced by cocaine

is not necessarily proof of transport into nerves as a mechanism termin-

ating the action of the sympathetic mediator. It is quite likely that

some amine could be returned to intraneuronal stores before diffusing

from the point of release to the effector cells. Inclusion of this por-

tion of released amine in the total recovered after cocainization would

lead to overestimation of the importance of nerve uptake in termination

of action.

In addition, an increased effluent output of noradrenaline or

a decreased tissue uptake of sympathomimetic amines produced by cocaine

should not be automatically considered solely due to blockade of nerve

uptake processes, although this seems to be a common practice. The ex-

periments reported in this thesis suggest that cocaine also impairs the

capacity of effector cells to inactivate amines. This was most clearly

demonstrated in experiments with phenylephrine, where it was found that

MAO and the cocaine sensitive mechanism did not function totally inde-

pendently, and the overlap could not be adequately explained on the basis of intraneuronal MAO. An overlap of the effects of COMT and of the cocaine sensitive mechanism was also observed in experiments with adrenaline. (See discussion in Section V, H.)

Indications of an action of cocaine on effector cells can also be found in previous reports by others. If the blockade of tissue uptake of exogenous catecholamines is solely due to blockade of uptake by nerves, cocaine should divert amine to metabolic disposition in other cells, i.e., a decreased "uptake" of noradrenaline should be associated with an increased production of metabolites. However, when metabolite levels have been reported, they are usually unchanged or decreased. Van Zwieten et al. (1965) examined the effect of cocaine on the radioactive material in tissues 2 minutes after the injection of H^3-noradrenaline or adrenaline. A careful analysis of their results reveals that not only were the concentrations of these amines in the heart and spleen lower in cocaine treated than in control animals, but that metanephrine levels were unchanged and normetanephrine levels in heart and plasma were decreased without any corresponding increase in the deaminated, O-methylated metabolite. Similar unexplained findings of decreased levels of metabolites in cocaine pretreated animals after H^3-catecholamine administration have been reported by Hertting et al. (1961b).

The results presented in this thesis clearly indicate that metabolism can compete effectively with storage and binding as a means of terminating the action of catecholamines, and the failure to find increased metabolism in the studies mentioned above suggests that cocaine may interfere with the access of amines to sites of metabolic inactiva-

tion in nonnervous (effector) cells. Indeed, Samorajski et al. (1964)

found a decreased concentration of total radioactivity in effector cells

of cocaine pretreated mice 2 minutes after the administration of triti-

ated noradrenaline, by an autoradiographic technique. Other evidence

for an action of cocaine on effector cells which results in impaired

binding of the transmitter is found in the observations of Kopin (1964)

that although H^3-noradrenaline taken up by denervated rat salivary glands

is not depleted by reserpine, it is released by tyramine, and that cocaine

interferes "with entry of the labelled catecholamine into this storage

site." (See also Fischer et al., 1965.)

Trendelenburg (1965) found that the bulk of intraportally in-

jected noradrenaline and adrenaline was inactivated metabolically in pas-

sage through the liver. However, cocaine produced a statistically signi-

ficant decrease in the hepatic inactivation of H^3-adrenaline and an al-

most significant decrease in the inactivation of H^3-noradrenaline. A

comparison of his results with MAO and COMT inhibitors and with cocaine

indicates that the action of the latter cannot be completely independent

of metabolic inactivation. In this connection it may be recalled that

Van Zwieten et al. (1965) found that the level of circulating normetan-

ephrine 2 minutes after the injection of H^3-noradrenaline was decreased

by cocaine. O-methylation in the liver is the major fate of circulating

noradrenaline in rodents (Crout et al., 1961; Carlsson and Waldeck, 1963).

The results of experiments reported in this thesis and a number

of otherwise anomalous published observations can be explained on the

basis that cocaine can block the movement of certain amines both into

nerves and into extraneuronal tissue cells. Blockade of the latter,

which may vary in degree from one organ system to another, probably contributes to the reported increase in effluent noradrenaline after stimulation of sympathetic nerves in the presence of cocaine.

The β-haloalkylamine adrenergic blocking agents Dibenamine and phenoxybenzamine appear to elevate the venous output of noradrenaline from the cat spleen more markedly and consistently than does cocaine (Brown and Gillespie, 1957; Brown et al., 1961; Bacq et al., 1960; Blakeley et al., 1963; Kirpekar and Cervoni, 1963; cf. Thoenen et al., 1963). For example, Kirpekar and Cervoni (1963) reported that the output of noradrenaline after stimulation of the splenic nerves at a frequency of 10/sec. was increased from 0.25 to 0.4 ng/stim. by cocaine and to 0.85 ng/stim. by phenoxybenzamine. This effect of the β-haloalkylamines was initially considered to reflect an important role of α adrenergic receptors in the disposition of adrenergic mediator. However, with the emergence of the concept that the major action of cocaine on amine disposition is inhibition of uptake by nerve cells, an action similar to that of cocaine was attributed to the β-haloalkylamines, and a majority of workers in the field of adrenergic mechanisms now consider their effects an effluent output of noradrenaline to be confirmation of the major role of uptake into nerves in termination of the action of the adrenergic mediator.

The present experiments showed that although the predominant effect of cocaine is blockade of processes leading to the binding and storage of sympathomimetic amines, that of the β-haloalkylamine GD-131 is to block their access to sites of enzymatic degradation in effector cells. GD-131 affected binding and storage of amines, the cocaine sen-

sitive mechanism, only after the maximal exposure which could be studied, just short of that producing α adrenergic blockade. Whether the relative prominence of these two actions is constant or varies widely, as does α adrenergic blocking activity, within the series of β-haloalkylamines, cannot be assessed from information now available. However, it is apparent that the increased effluent output of noradrenaline after nerve stimulation and the decreased tissue "uptake" of sympathomimetic amines produced by β-haloalkylamine adrenergic blocking agents is unrelated to the blockade of α adrenergic receptors. The major part of these effects is probably due to the action inhibiting access to sites of metabolic inactivation, predominantly in effector cells, with perhaps a lesser contribution of the action preventing access to binding and storage sites, predominantly, but not exclusively, in adrenergic nerves. The lesser effect of cocaine on effluent output of noradrenaline is due predominantly, but not exclusively, to blockade of processes leading to amine binding and storage.

The expression of these drug actions is dependent upon the processes normally involved in the disposition and termination of action of noradrenaline and of other sympathomimetic amines. The hypothesis regarding mechanisms terminating the action of the adrenergic mediator proposed in this thesis indicates that a considerable portion of the noradrenaline released by sympathetic nerves diffuses to the effector cells and passes through their plasma membranes to intracellular sites of metabolic inactivation, rather than being returned to storage in nerves, as is assumed by the current dominant concept. These metabolites should ultimately appear in the venous effluent from the effector organ.

A typical study of the fate of mediator released by nerve stimulation is that of Hertting and Axelrod (1961), who reported a marked elevation in the venous output of noradrenaline after stimulation of the sympathetic nerves to the cat spleen at a frequency of 10/sec. and a lesser, but significant, elevation in the output of normetanephrine. Similar findings were reported by Rosell et al. (1963) after stimulation of the nerves to a skeletal muscle vascular bed in the cat at frequencies of 5 to 12/sec. They found a predominance of metabolites in the resting venous outflow, but noradrenaline was predominant after nerve stimulation. After priming with H^3-noradrenaline, Chidsey et al. (1963) found that only 13 to 25% of the radioactivity in the coronary sinus blood of dogs was due to unaltered amine and that 39 to 56% was due to normetanephrine. The administration of tyramine, in an attempt to stimulate nerve activity, increased the quantity of noradrenaline much more than that of normetanephrine.

These results demonstrate that enzymatic degradation plays a role in the disposition of noradrenaline released by nerve stimulation, as required by the proposed hypothesis. However, it is possible that they underestimate the portion of released mediator undergoing metabolism. The actual amount of mediator liberated by depolarization of sympathetic nerve terminals is unknown. Workers who believe that uptake by nerves is the major mechanism terminating the action of the mediator assume that, in the presence of cocaine, the amount appearing in the perfusing medium is virtually the total liberated, and that the increase due to cocaine is solely a result of blockade of uptake by nerves. This appears to be most unlikely. (See discussion under 3), above.)

For the noradrenaline and metabolites measured in the effluent during and shortly after stimulation of sympathetic nerves to a perfused organ to reflect accurately the fate of the mediator released, mobility in all of its various states (e.g., O-methylated, receptor-exciting, etc.) and from varying intra- and extracellular loci in the nerve-effector complex must be equal, so that all are equally represented in the effluent. Such a situation is most improbable. Instead one would expect the early peak of unchanged amine in the effluent to represent "excess" in the extracellular space which has not penetrated the diffusion barrier between this and the environment of tissue receptors (Section III, A, 1), and has not been exposed to endogenous mechanisms of inactivation. This is supported by the observation that, in the absence of drug treatment, the amount of noradrenaline per stimulus recovered in the effluent increases with frequency over a considerable range (Brown and Gillespie, 1957). The amount recovered during stimulation at physiological frequencies is almost undetectable except by tracer techniques.

Experiments discussed above demonstrate the capacity of effector cells to hold exogenous catecholamine for prolonged periods of time, either unchanged or as metabolites, and several published observations suggest that the mobility of the latter in tissues may be considerably less than that of noradrenaline. This is a logical explanation for the report that although the output of normetanephrine from the heart was increased less than that of noradrenaline by tyramine, the latter declined toward normal over a period of 30 minutes, whereas the former showed no tendency to decline even after 45 minutes (Chidsey et al., 1963). Differences in mobility would also explain the finding of Anden

et al. (1963) that 30 minutes after the administration of H^3-noradrenaline the chronically denervated salivary glands of reserpinized rats contained a negligible concentration of labelled noradrenaline, but a high concentration of normetanephrine.

The above representative observations on the output of noradrenaline and metabolites from organs showed the latter to be equal to or greater than the former in resting venous outflow, whereas predominantly noradrenaline was found during and shortly after nerve stimulation. The composition during nerve stimulation has been taken as evidence of a minor role of metabolism in the inactivation of mediator released by nerve activity. However, in view of the probable major differences in the mobilities of different components, this transient may present a much less accurate picture of physiological events than does the resting venous outflow, which may approach a steady state system. In addition, it is quite possible that the frequencies of stimulation used, at or above the maximal physiological rates of sympathetic nerve firing (Celander, 1954), and the administration of tyramine release an excess of mediator which does not enter the pathways of normal physiological disposition.

Combined inhibition of COMT and MAO does not increase effluent output of noradrenaline during and shortly after sympathetic nerve stimulation, whereas pretreatment with a β-haloalkylamine does (Brown, 1960). However, experiments presented in this thesis demonstrated that a β-haloalkylamine (GD-131) and inhibition of the two enzymes have the same ultimate effect on noradrenaline disposition, presumably because the former prevents access to the latter. The major effect of GD-131 is most probably at some nonnervous cellular or intracellular membrane, and it is

at this point that the distinction between appearance in the effluent and endogenous inactivation probably occurs.

It can be considered that membrane penetration and distribution within cell water is the immediate event which terminates the action of noradrenaline. However, the capacity of this process is obviously limited, and it probably would contribute little to body economy without the associated endogenous processes for more definitive inactivation of the mediator. These processes and their relative contributions to the inactivation of noradrenaline, as determined by the oil immersion technique, are depicted diagramatically in figure 53a, as are the areas of endogenous inactivation inhibited by cocaine and GD-131. Conversion to pharmacologically inactive products by COMT and MAO in the effector cells is the ultimate fate of a major part of the amine, but the rate at which this occurs would not immediately affect adrenergic nerve function as long as it was adequate to maintain a steady state with a low intracellular level of free amine. O-methylation appears to be the dominant primary enzymatic step in the inactivation, but its role is largely overlapped by the effective alternative pathway of deanimation. Binding and storage of amine, involving both neuronal and extraneuronal sites, appears to play a smaller role in adrenergic mediator inactivation than do the enzymatic processes. Access to sites of binding and storage, both neuronal and extraneuronal, and to sites of enzymatic inactivation appears to differ somewhat, and can be differentiated by the effects of cocaine and GD-131.

Processes contributing to the potentiation of responses to sympathomimetic amines by cocaine and GD-131 are presented diagramatically

in figure 53b for comparison with the processes involved in inactivation. It can be seen that there is considerable overlap of the two in the case of GD-131, but very little for cocaine.

A model for the proposed theory of the termination of action of the sympathetic mediator is presented in figure 54. A charge of noradrenaline is released by depolarization of an adrenergic nerve terminal at "A". Part of this is transported back into nerve cells (B), where it is restored or deaminated, before leaving the plexus of adrenergic nerves to reach the effector cells. This portion does not participate in "chemical mediation" and, therefore, is not involved in termination of action. A sizeable portion of the released mediator diffuses to the vicinity of effector cells (E), with variable loss into the circulation. Amine which reaches the environment of the appropriate tissue receptors (R), which appear to lie within or behind a diffusion barrier (DB) associated with the plasma membrane (PM) of effector cells has accomplished chemical mediation, and its inactivation identifies the mechanisms of termination of mediator action.

The terminating mechanism for a major portion of this amine is penetration of diffusion barriers (membranes) at the surface of or within (M) effector cells, followed by either metabolic alteration or binding. COMT appears to be the primary inactivating enzyme, with MAO providing an effective alternative pathway. This relationship may be determined by some anatomical arrangement of the enzymes such that the amine entering the cell passes areas of COMT activity before reaching sites of deamination, predominantly in mitochondria. Diffusion away from the effector cells into the circulation or to reincorporation in

nerves may terminate the action of or provide later disposition for

some portion of the mediator.

Fig. 54. Model Depicting Mechanisms Terminating the Action of the

Sympathetic Mediator.

"A" indicates the site of release of a charge of

noradrenaline by nerve depolarization, and "B" sites of re-

uptake. "P" is the plasma membrane of an effector cell (E)

and associated diffusion barrier (DB), behind at least part

of which the α adrenergic receptors (R) are located. M's

represent intracellular membranes or other barriers to dif-

fusion not morphologically defined. The enzymes COMT and

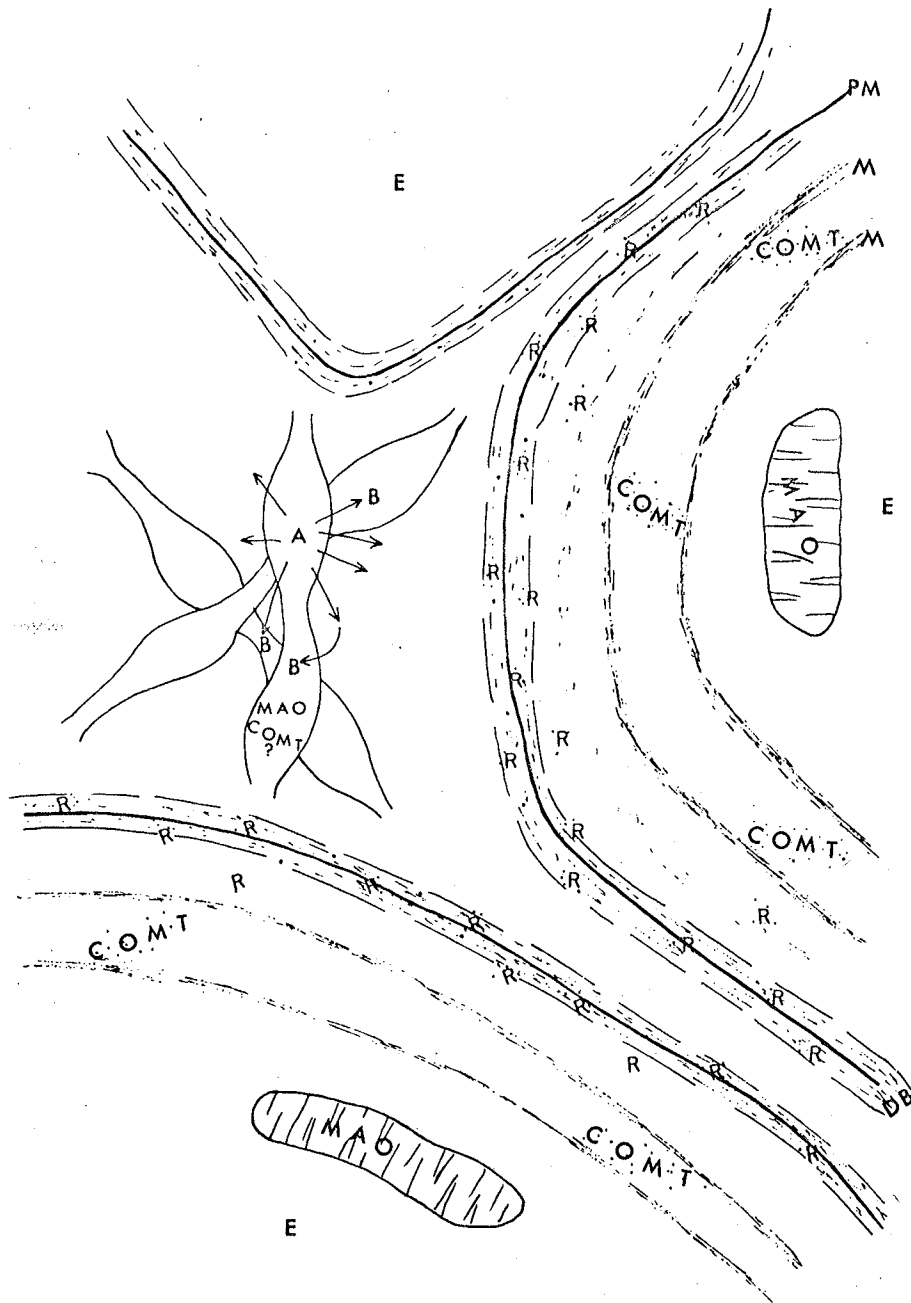MAO are in cell cytoplasm and mitochondria, respectively.

Fig. 54.   Model Depicting Mechanisms Terminating the
Action of the Sympathetic Mediator.

IX. SUMMARY AND CONCLUSIONS

It was demonstrated that the gradual relaxation of contractions of vascular smooth muscle after the washout of stimulant drugs in aqueous media in vitro, is related to the gradually decreasing concentration of "active" drug in the vicinity of the appropriate tissue receptors. This was suggested by the observation that rates of relaxation from contractions produced by various α adrenergic stimulants differed, and that the rate of relaxation could be increased by non-competitive interference with drug-induced tone. Experiments with enzyme inhibitors and the technique of receptor protection confirmed that the relaxation of rabbit aortic strips could be correlated with a gradually declining concentration of agonist in the biophase of the tissue.

These results indicated that relaxation after a drug-induced contraction could be used as a measure of the termination of drug action, but to equate this with the effects of endogenous mechanisms of inactivation it was necessary to eliminate loss by diffusion into the surrounding medium. This was accomplished by developing a technique of oil immersion, in which the aqueous medium in the muscle chambers was replaced by mineral oil after the tissue had reached equilibrium, or a steady state, with the drug under study. It was demonstrated that the oil per se exerted no pharmacological action, and that it did not interfere with tissue function or the exchange of gases between the tissue and its environment, as reflected in contractile performance. The selective slowing by iproniazid of relaxation after contractions produced by dopamine, a substrate for monoamine oxidase (MAO), but not by

a nonsubstrate, Cobefrine, indicated that the technique is capable of assaying the activity of individual endogenous mechanisms when used in combination with specific inhibitors of possible inactivation pathways.

Experiments with the oil immersion technique demonstrated that the primary mechanism for the enzymatic inactivation of both noradrenaline and adrenaline in aortic tissue is O-methylation. Inhibition of catechol-O-methyl transferase (COMT) impaired the rate of inactivation of a low concentration of noradrenaline considerably less than it did that of the same concentration of adrenaline. Inhibition of monoamine oxidase (MAO) had a negligible effect on the inactivation of either, if COMT activity was unimpaired. Comparison of the increases in relaxation time due to various combinations of inhibitors of inactivation pathways indicated that COMT and MAO function as if they were arranged anatomically in series, with the latter an effective alternate mechanism for the inactivation of noradrenaline and somewhat less effective for adrenaline. Experiments with a 100-fold higher concentration of these catecholamines showed that the major endogenous pathways of inactivation were deamination and O-methylation for noradrenaline and adrenaline, respectively. It was suggested that the high concentrations of agonist swamped the organized, anatomically arranged system for their inactivation, and resulted in a more or less simultaneous presentation of amine to both enzymes, revealing their relative capacities for handling the two catecholamines.

Enzymatic processes were found to make a considerably more important contribution than binding and storage mechanisms as assessed on the basis of the effects of cocaine and methylphenidate, to the

inactivation of low concentrations of noradrenaline and adrenaline, and to account for almost all of the inactivation of high concentrations.

The roles of various endogenous mechanisms in terminating the action of sympathomimetics, as determined by the technique of oil immersion, were compared with those assigned by the traditional method based on potentiation of responses to agonists. It was found that the latter can be grossly inaccurate, both in detecting major endogenous inactivating mechanisms and in quantitating their contributions to the termination of action. This appears to be because of the complication introduced by diffusion into the surrounding medium. This factor may be of particular importance where the interaction of two or more endogenous mechanisms is involved, as in the "series" arrangement of COMT and MAO.

Other experiments demonstrated that the presence of potentiation per se cannot be equated with effects on inactivation, either qualitatively or quantitatively. In particular, most of the potentiation produced by cocaine appeared to be unrelated to blockade of inactivation by transport into nerves. Using cocaine and GD-131, a β-haloalkylamine congener of Dibenamine and phenoxybenzamine with little α adrenergic blocking activity, as potentiating agents, the following evidence for this dissociation was obtained.

1) Potentiation of responses to phenylephrine by cocaine was unaltered after a 60 minute exposure of reserpinized, iproniazid pretreated aortic strips to this agonist. In the absence of both intraneuronal storage and metabolism, net uptake of amine by nerves should

have been markedly reduced, with a concomitant reduction in the poten-
tiation produced by cocaine if this were a result of blockade of nerve
membrane transport of amine.

2) Cocaine and methylphenidate potentiated some responses to amines
(histamine and 5-hydroxytryptamine) which appear not to be taken up and
stored in adrenergic nerves. Methylphenidate was tested for and found
to have no effect on the disposition of histamine.

3) Cocaine still effectively potentiated responses of aortic
strips to noradrenaline after their neuronal elements had been allowed
to degenerate during prolonged periods in the cold and at 37°C.

4) Both cocaine and GD-131 effectively potentiated responses of
aortic strips to methoxamine, although studies by the oil immersion
technique confirmed the complete absence of endogenous mechanisms for
the inactivation of this sympathomimetic.

5) Both procaine and cocaine virtually eliminated the residual
inactivation of phenylephrine in iproniazid pretreated strips, but only
the latter potentiated responses to this amine. Cocaine still effect-
ively potentiated responses to phenylephrine in the presence of procaine.

6) The increased concentrations of active amine in the vicinity of
tissue receptors which could result from the delay in inactivation pro-
duced by cocaine or GD-131 were inadequate to account for and were
poorly correlated with the potentiation produced by these agents.

A detailed comparison of the two potentiating agents, cocaine
and GD-131 indicated that their major effect was exerted through a
common mechanism but that a small part of the effect of cocaine, and up
to nearly half of that of GD-131 involved independent mechanisms, related
to their effects on mechanisms of inactivation. Cocaine and GD-131

produced almost identical potentiation of responses to methoxamine, an effect entirely unrelated to inactivation. When added in sequence, cocaine produced a minor, but reproducible, potentiation of responses to noradrenaline in the presence of GD-131, whereas GD-131 caused a reduced, but still considerable, potentiation in the presence of cocaine. In addition, inhibition of COMT markedly decreased the potentiation of responses to tyramine in iproniazid pretreated aortic strips by GD-131, and appeared to decrease those in otherwise untreated noradrenaline contracted strips. Inhibition of COMT also somewhat decreased the potentiation produced by maximal doses of cocaine. GD-131 potentiated responses to noradrenaline significantly more than did the maximally effective concentration of cocaine.

The results obtained with strips from reserpinized animals did not support the hypothesis that decreased inactivation due to inhibition of storage mechanisms is responsible for the potentiation of responses to certain sympathomimetic amines by reserpine. In fact, reserpinized strips tended to inactivate both noradrenaline and phenylephrine more rapidly than did control preparations. Reserpinized preparations were found to have a decreased efficiency of storage and binding of noradrenaline, with a diversion of amine to metabolic inactivation, but the diversion was to COMT, rather than to MAO as is currently believed.

Both cocaine and GD-131 were found to alter the endogenous disposition of sympathomimetic amines, but the major mechanisms involved were different. The effect of cocaine on inactivation of amines appeared to be primarily due to blockade of binding and storage, with a

lesser action inhibiting access of amines to sites of metabolic inactivation. Although not an enzyme inhibitor, the major effect of GD-131 on amine inactivation was to "simulate" enzyme inhibition, apparently by preventing access of amines to the enzymes. It blocked access to sites of binding and storage only at the maximal exposure short of producing α adrenergic receptor blockade. The major evidence adduced to support these mechanisms of action were as follows:

1) Impairment of the inactivation of noradrenaline by moderate exposure to GD-131 (1×10^{-5} for 10 to 15 min.) and by cocaine (1×10^{-4}) was approximately additive.

2) Moderate exposure of aortic strips to GD-131 reduced the rate of inactivation of noradrenaline more than did inhibition of either MAO or COMT.

3) Cocaine produced a further reduction in the rate of inactivation of noradrenaline by aortic strips in which both COMT and MAO had been inhibited, but moderate exposure to GD-131 did not, indicating that the major action of the latter is on access of the amine to sites of enzymatic degradation and not on transport to sites of binding and storage.

4) GD-131 potentiated responses to tyramine, whereas cocaine inhibited them. This provided further evidence that the major action of GD-131 is not on nerve membrane transport of amine to storage sites.

5) Maximal exposure to GD-131 short of producing α adrenergic blockade sometimes inhibited the inactivation of noradrenaline as much as did combined treatment with iproniazid, tropolone and cocaine, which indicates that this compound has also the ability to block the binding

and storage of amines, as does cocaine.

6) Cocaine appeared to have minor components of action comparable
to the major properties of GD-131. It produced a smaller decrease in
the inactivation of phenylephrine and adrenaline in aortic strips in
which MAO and COMT, respectively, had been inhibited than it did in
the controls, indicating some overlap with the major pathway of amine
metabolism in each case.

Experiments performed with tyramine, bretylium and guanethidine
indicated that these agents all cause release of catecholamines from
endogenous stores in concentrations producing no more than minimal re-
sponses in Krebs medium. The amplitude of contraction of strips ex-
posed to any one of these agents increased after oil immersion to reach
a plateau, which was sustained for the duration of oil immersion.
Phenoxybenzamine blocked this response and its magnitude and rate of
rise were markedly reduced in strips from reserpinized animals. The
barrier to diffusion produced by the oil made it possible to demonstrate
otherwise undetectable amounts of endogenously released noradrenaline.
This was demonstrated by the fact that reserpinized preparations treated
with any one of the above agents and unreserpinized strips treated with
iproniazid plus tropolone plus cocaine consistently responded with
gradual contractions in oil, although no response was visible in Krebs
medium. The response of unreserpinized strips treated with the 3 in-
hibitors was probably due to noradrenaline "spontaneously" released from
nerve endings, which was trapped by the oil and protected against endo-
genous inactivation by the combination of inhibitors.

The suitability of the technique of oil immersion for studies

of endogenous mechanisms of inactivation of agents other than sympatho-mimetic amines was demonstrated in experiments showing that MAO plays an important role in the inactivation of 5-hydroxytryptamine and that diamine oxidase and an additional mechanism, perhaps N-methyl trans-ferase, are responsible for the endogenous inactivation of histamine.

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