THE PHYSIOLOGICAL AND PHARMACOLOGICAL ACTIONS AND FATE OF SEVERAL SYMPATHOMIMETIC AMINES IN VASCULAR BEDS

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by

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DECLARATION

I declare that this thesis is my own composition and that it is a record of original work conducted during the years 1967, 1968, 1969 and 1970 in the Department of Human Physiology and Pharmacology, University of Adelaide.

The work described herein has not been submitted for any other degree, award or diploma and to the best of my knowledge this thesis contains no material previously published by any other person, except where due reference is made in the text.

The results have been presented in part to meetings of the Australasian Society of Clinical and Experimental Pharmacologists in 1967, 1968 and 1969 and to meetings of the Australian Physiological and Pharmacological Society in 1968 and 1969. In addition, a considerable amount of the material has been published or accepted for publication in the following journals:

Br. J. Pharmac., 36, 602-610 (1969)

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CHAPTER 1

GENERAL INTRODUCTION

It is the purpose of this thesis to examine further the physiological actions and fate of several sympathomimetic amines. Apart from the effects produced by such amines, emphasis has been placed on the mechanisms by which they are broken down in various tissues. As such, the role of monoamine oxidase in the reactions involving their metabolic degradation in vascular tissue has been closely examined.

Early history:

The metabolic fate of amines in the mammalian body has been the subject of investigation since the latter part of the 19th Century. Schmiedeberg (1877) produced the first clear evidence that amines are broken down in the animal by reactions involving deamination. He demonstrated in the dog that benzylamine, given orally, was excreted as the corresponding carboxylic acid (hippuric acid). It was recognized at this stage and later shown (Schmiedeberg, 1881) that this involved the intermediate formation of benzoic acid, which indicated that the benzylamine had been broken down *in vivo* with the formation of ammonia. Later, Minkowski (1883) showed that the deamination reaction also occurred *in vitro* by the production

of benzoic acid following incubation of minced rabbit tissues with benzylamine. Mosso (1889) studied benzylamine metabolism quantitatively by giving the amine subcutaneously to a dog, with more than 90% recovery as hippuric acid in the urine.

Ewins and Laidlaw (1910) investigated the metabolism of tyramine (p-hydroxyphenylethylamine) by perfusing this amine through the liver of the cat and rabbit. They showed that in the liver, the amine was largely converted into p-hydroxyphenylacetic acid. Ewins and Laidlaw (1913) also indicated that deamination was the metabolic pathway in the breakdown of tryptamine (β-indolethylamine) when they isolated indoleacetic acid as the degradation product of indolethylamine which had been perfused through the liver of the rabbit. Phenylethylamine is the next higher homologue of benzylamine, and the administration of phenylethylamine hydrochloride to a rabbit by mouth was associated with recovery of 87% of the theoretical amount of phenylacetic acid in the urine (Guggenheim and Löffler, 1916).

In all of the earlier studies on the metabolic fate of amines in tissues, it was assumed that deamination of an amine was an hydrolytic reaction involving the formation of an alcohol which was then further oxidized to the corresponding carboxylic acid. The possibility of oxidative deamination of amines, however, was not considered until the demonstration of an enzyme, tyramine

oxidase, by Hare (1928). She showed that cell-free extracts of the livers of various animals, including the rat, dog and sheep, oxidized tyramine to p-hydroxybenzaldehyde, hydrogen peroxide and ammonia. This was the first evidence that amines could be oxidatively deaminated in mammalian tissues, although at that time, the exact nature of the reactions involved was not completely clear.

It was later found (Pugh and Quastel, 1937a) that aliphatic amines could be oxidatively deaminated by various mammalian tissues, including the liver of the rat and guinea-pig and the brain of the rat. An enzyme (aliphatic amine oxidase), present in these tissues, was considered to be responsible for the changes observed. Another group of workers, Blaschko, Richter and Schlossmann (1937a) also described an enzyme (adrenaline oxidase) which was present mainly in the liver, kidney and intestine of the rat, guinea-pig and rabbit. They found this enzyme to be capable of actively destroying the biological activity of adrenaline.

Subsequently, however, from evidence presented by Kohn (1937), Pugh and Quastel (1937b), and Blaschko, Richter and Schlossmann (1937b), it was concluded that the enzymes referred to as tyramine oxidase, aliphatic amine oxidase and adrenaline oxidase, were almost certainly identical. In particular, Blaschko, Richter and Schlossmann (1937b) showed that the three enzymes were similar:

(a) in their distribution in mammalian tissues,

(b) in the chemical reactions which they catalyzed,

(c) in their behaviour towards inhibitors, and

(d) in that their substrates competed with each other and did not show additive effects.

It was proposed that this single enzyme be designated monoamine oxidase (Zeller, Stern and Wenk, 1940; Zeller, 1941) in order to differentiate it from diamine oxidase which was known to oxidize a number of aliphatic diamines. Diamine oxidase was originally referred to as histaminase because it was first reported as the enzyme responsible for the inactivation of histamine in mammalian tissue (Best and McHenry, 1930). This followed the work of Best (1929) who demonstrated that finely-minced mammalian lung tissue which was suspended in physiological saline and incubated in the presence of toluene at 37°C, caused the disappearance of naturally-occurring or added histamine. Subsequently it was realized that this enzyme oxidized a number of diamines such as putrescine and cadaverine more readily than it oxidized histamine and so the name "diamine oxidase" was thus suggested (Zeller, 1938). However, this differentiation between monoamine oxidase and diamine oxidase is somewhat illusory as it was later shown that monoamine oxidase acted upon a great number of diamines (Blaschko and Duthie, 1945a; Blaschko and Hawkins, 1950) and a number of monoamines were

found to be substrates of diamine oxidase (Fouts, 1954; Fouts, Blanksma, Carbon and Zeller, 1957).

Since the early discoveries of Hare (1928) and Best (1929), many enzymes have been found to catalyze the oxidative deamination of a wide range of amines and diamines. The members of this group of "amine oxidases" include animal, plant and bacterial enzymes. The different amine oxidases vary in their general distribution in that they may be located in mitochondria of the cells, in the soluble components of tissues or in blood plasma. They have shown varied species, substrate and inhibitor specificity (Alles and Heegaard, 1943; Pratesi and Blaschko, 1959; Blaschko, Friedman, Hawes and Nilsson, 1959) and attempts to classify them, based on their substrate specificities, have not been very profitable. However, Blaschko, Friedman, Hawes and Nilsson (1959) classified them in relation to differences in their inhibitor specificities and this enabled two broad groups to be differentiated.

Enzymes in the first group were inhibited by carbonyl reagents such as hydrazine, semicarbazide and cyanide. Members of this group included diamine oxidase (Zeller, 1951); mescaline oxidase of rabbit liver (Bernheim and Bernheim, 1938); spermine oxidase in ruminant plasma (Hirsch, 1953); benzylamine oxidase in non-ruminant plasma (Bergeret, Blaschko and Hawes, 1957); plant amine oxidase (Kenten and Mann, 1952) and a bacterial enzyme (Weaver

and Herbst, 1958). The second group consisted of the enzymes which were not inhibited by carbonyl reagents. This group included the amine oxidase (tyamine oxidase) first described by Hare (1928) and which was subsequently referred to as monoamine oxidase (Zeller, Stern and Wenk, 1940; Zeller, 1941). Such a classification as this cannot be complete and, in particular, it is emphasized that terms such as "diamine oxidase" and "monoamine oxidase" do not apply to single entities but to collections of enzymes, respectively, with different properties, including substrate specificities. The main purpose of this thesis has been the investigation of the role which monoamine oxidase plays, particularly in vascular tissue. Accordingly, subsequent discussion is concerned mainly with this enzyme group.

It has been known for many years that amine oxidase (monoamine oxidase) acted *in vitro* on aliphatic amines which had an amino group attached to a terminal carbon atom (Blaschko, Richter and Schlossmann, 1937b); for example, compounds of the type RCH_2NH_2. In the oxidation of a number of primary, secondary and tertiary amines by amine oxidase (monoamine oxidase), Richter (1937) demonstrated the formation in each case of either an aldehyde and ammonia or a lower amine. The quaternary salt, N-methylhordenine chloride was not oxidized by the enzyme. It had also been shown, for the substrates tyramine, adrenaline and the aliphatic amines,

that hydrogen peroxide was formed as a reaction product (Hare, 1928; Philpot, 1937; Pugh and Quastel, 1937a). However, the hydrogen peroxide produced was normally destroyed by catalase in the experimental enzyme preparations.

Bernheim (1931) provided evidence that the respective aldehyde formed by amine deamination was further oxidized to the corresponding carboxylic acid. Her data suggested that the oxidative deamination of tyramine by tyramine oxidase (monoamine oxidase) involved the formation of an aldehyde which was then oxidized directly to the corresponding acid (p-hydroxyphenylacetic acid). Weissbach, Redfield and Udenfriend (1957) also demonstrated that the action of monoamine oxidase on serotonin was to convert it to p-hydroxyphenylacetic acid via the intermediate product, 5-hydroxyindoleacetaldehyde. These latter studies indicated that the aldehyde was converted to the acid by means of aldehyde dehydrogenase linked with diphosphopyridine nucleotide.

Monoamine oxidase has a wide distribution in mammalian tissue. It is present biochemically in highest concentration in the liver, kidney and intestine of most animals examined, but most organs showed some activity, including nervous tissue, lungs, salivary glands, adrenal glands, thyroid, pancreas, smooth muscle and heart in some species (Bhagvat, Blaschko and Richter, 1939; Blaschko, 1952). Thompson and Tickner (1951) found monoamine oxidase

activity in many of the blood vessels of the rabbit, including those of the ear; however, the veins had a much lower content of the enzyme than the arteries examined. Skeletal muscle and blood contained little or no activity (Blaschko, 1952). A noticeable finding in these studies, however, was that there were considerable species differences in the enzyme content of identical organs. These biochemical results were largely confirmed by the histochemical investigations of Koelle and Valk, Jr (1954), and Glenner, Burtner and Brown, Jr (1957) who similarly found a very wide distribution of monoamine oxidase in the various tissues and organs of different mammalian species.

Studies on the subcellular and submitochondrial localization of monoamine oxidase in mammalian liver and other tissues have revealed it to be usually a particulate and relatively insoluble enzyme which is largely associated with mitochondria. The procedures employed in these investigations have included differential centrifugation of tissue homogenates, other biochemical separation techniques and also electron microscopy of submitochondrial particles. Certain investigations involving homogenates of rat liver (Cotzias and Dole, 1951), bovine adrenal medulla (Rodriguez de Lores Arnaiz and De Robertis (1962) have supported the premise that monoamine oxidase is either predominantly or exclusively a mitochondrial enzyme. However, other studies have suggested that variable amounts of the enzyme occurred

in microsomal fractions as well as in mitochondrial fractions on differential centrifugation of tissue homogenates. For instance, Hawkins (1952) found that in homogenates of rat liver, only about two-thirds of the total monoamine oxidase activity was associated with the crude mitochondrial fraction whilst the remainder was present with the non-mitochondrial particulate microsomal fraction. Similarly, the studies of Oswald and Strittmatter (1963), employing rat liver homogenates, also suggested a dual intracellular location of the enzyme, with the major portion in the mitochondria and a smaller but significant portion sedimenting with the microsomal fraction.

The complexity of the mitochondrion, which consists of two membrane systems and at least two compartments, has accentuated many of the difficulties which have occurred in relation to the precise location of its enzymes and chemical components by means of submitochondrial fractionation techniques and electron microscopy. There have been recent attempts to separate the two membranes in order to locate the position of the monoamine oxidase molecule (Schnaitman, Erwin and Greenawalt, 1967). However, one of the major problems encountered was that the inner membrane was noted to be extremely folded, to form cristae which could comprise 75% or more of the total mitochondrial membrane. Hence, care had to be taken in obtaining preparations of the outer membrane which were not heavily

contaminated with the inner membrane.

Recent experimental evidence, as indicated by the following studies, has favoured a location of the monoamine oxidase molecule in the region of the outer membrane of the mitochondrion. For example, Schnaitman, Erwin and Greenawalt (1967), using subfractions of rat liver mitochondria and electron microscopy, showed that the enzyme was located on the isolated outer mitochondrial membrane. They noted also that some vesiculation of the outer membrane occurred during the technical procedure and they suggested that such artifacts of homogenization might have accounted for the previous reports of monoamine oxidase in the so-called microsomal fraction of the homogenates. The biochemical experiments of Tipton (1967) with rat liver and brain showed that approximately 75% of liver and more than 50% of brain mitochondrial monoamine oxidase activity appeared in the outer membrane fraction. This author also demonstrated that the inner mitochondrial membrane was impermeable to several substrates of the enzyme. In addition, the histochemical studies of Boadle and Bloom (1969), involving electron microscopical examination of guinea-pig cortex, suggested some relationship of the enzyme with the outer mitochondrial membrane and the space between the inner and outer membranes.

Other studies, incorporating separation techniques and electron microscopy, seemed to imply that there was a close

relationship between monoamine oxidase and the noradrenaline storage granules in homogenates of various sympathetically-innervated mammalian tissues. For example, the experiments of Roth and Stjärne (1966) with homogenates of bovine splenic nerve, indicated that the nerve granule fraction contained substantial monoamine oxidase activity. It was also noted by electron microscopy that this fraction was largely free of mitochondria, which seemed to infer that the monoamine oxidase which followed the nerve granules on centrifugation was not present in normal mitochondria. In addition, experiments with homogenates of rat heart showed that approximately 50% of the total monoamine oxidase activity was present in smaller microsomal particles, with only 25% in mitochondria (de Champlain, Krakoff and Axelrod, 1968) and the microsomes sedimented with the noradrenaline storage granules when examined by continuous sucrose gradient techniques (de Champlain, Axelrod, Krakoff and Mueller, 1968).

Jarrott and Iversen (1968) investigated this possible association of monoamine oxidase and noradrenaline storage granules by examining homogenates of rat vas deferens and rat liver. They found that after differential centrifugation, 24% of the total monoamine oxidase activity of rat liver homogenates and 42% of that in vas deferens was recovered in the microsomal fractions, the remainder being recovered in a mitochondrial pellet. However, in the vas

deferens, which is a smooth muscle tissue with a rich sympathetic innervation, they were able to clearly separate the small low density "microsomal" particles containing monoamine oxidase from the more dense noradrenaline storage granules. They also demonstrated the presence of the monoamine oxidase-containing particles in sympathetically-denervated vas deferens in which the noradrenaline storage particles could no longer be detected. These authors therefore suggested that there was not likely to be any association of monoamine oxidase with noradrenaline storage particles in the vas deferens or elsewhere in the sympathetic nervous system. This conclusion was supported by the previous findings of Potter and Axelrod (1963a) who reported that there was little or no monoamine oxidase activity associated with noradrenaline storage particles which had been isolated from rat heart homogenates. Jarrott and Iversen (1968) also considered the question of the origin of the "microsomal" monoamine oxidase activity in the homogenates of rat liver and rat vas deferens. In accordance with the findings of Schnaitman, Erwin and Greenawalt (1967) they concluded that "microsomal" monoamine oxidase was the result of artifactual damage to the outer mitochondrial membrane during homogenization.

Studies on the homogeneity of monoamine oxidase have clearly shown that the enzyme displays great diversity of substrate specificity and inhibitor sensitivity in different tissues of the

same species and in identical organs and tissues of different mammalian species (Bhagvat, Blaschko and Richter, 1939; Alles and Heegaard, 1943; Pratesi and Blaschko, 1959; Hope and Smith, 1960). Oswald and Strittmatter (1963) suggested that such differences may reflect (a) intrinsic differences in monoamine oxidase of diverse origin, (b) activity of other enzymes or (c) differences in accessibility of substrate or inhibitor to the enzyme. The behaviour of monoamine oxidase towards inhibitors has, however, been more consistent than its behaviour towards different substrates and this led to a classification of amine oxidases (monoamine oxidases) in terms of their susceptibility towards inhibitors (Blaschko, Friedman, Hawes and Nilsson, 1959).

Carbonyl reagents such as cyanide and semicarbazide do not inhibit monoamine oxidase (Blaschko, 1952; Blaschko, Friedman, Hawes and Nilsson, 1959) and this is an important characteristic of the enzyme. Amines in which the amino group is not attached to a terminal carbon atom are not oxidized by monoamine oxidase at a measurable rate; however, they act as competitive inhibitors of the enzyme. Such inhibitors include ephedrine (Blaschko, 1938) and amphetamine (Mann and Quastel, 1940); however, amphetamine was found to be a better inhibitor than ephedrine. Many amidines are inhibitors of monoamine oxidase (Blaschko and Duthie, 1945b), although inhibition of the enzyme by these compounds does not seem to be of

a simple competitive nature. Many other compounds inhibit the enzyme to varying degrees and these include percaine, cocaine, procaine and other local anaesthetics (Philpot, 1940).

The discovery of the potent inhibitory properties of iproniazid (Zeller, Barsky, Fouts, Kirchheimer and Van Orden, 1952; Zeller and Barsky, 1952) stimulated the search for other powerful inhibitors of monoamine oxidase. In subsequent years, many new and potent inhibitors have been discovered and they cover a wide range of chemical classes. Three main groups have been described (Rand and Trinker, 1966) with other additional enzymes which are not included in these groups.

The first group, the hydrazine derivatives (general formula R_1-NH-NH-R_2), includes iproniazid and nialamide. Iproniazid exemplifies the monoamine oxidase inhibitors which are derivatives of hydrazine and the other members of the group are related to iproniazid. Although iproniazid and the respective substrate of monoamine oxidase initially compete for the enzyme, the action of iproniazid soon becomes non-competitive and irreversible (Pletscher, 1966). Hydrazine derivatives such as iproniazid (Zeller, Barsky and Berman, 1955; Davison, 1957; Taylor, Wykes, Gladish and Martin, 1960) and also certain non-hydrazine compounds such as pargyline cause maximum inhibition *in vitro* only after aerobic pre-incubation with the enzyme in the absence of the substrate. This increase in

inhibitory power with pre-incubation is possibly due to their transformation into compounds which represent the actual inhibitors reacting with the active centre of the enzyme (Pletscher, 1966). The active metabolite of iproniazid, for example, has been shown to be a volatile compound which may possibly be a product of the further metabolism of isopropylhydrazine (Kory and Mingioli, 1964). Nialamide, another member of this group, has also been shown to be a potent inhibitor of monoamine oxidase, both *in vivo* and *in vitro* (Rowe, Bloom, P'an and Finger, 1959a and b).

The second group consists of the harmala alkaloids, for example, harmine and harmaline, which behave as short-acting reversible inhibitors (Udenfriend, Witkop, Redfield and Weissbach, 1958). The third major group is comprised of monoamine oxidase inhibitors of the amphetamine type which are also indirectly-acting sympathomimetic amines. There is considerable evidence that amphetamine (Axelrod, Gordon, Hertting, Kopin and Potter, 1962; Trendelenburg, Muskus, Fleming and Gomez Alonso de la Sierra, 1962a and b) releases noradrenaline from noradrenergic storage sites in the same manner as tyramine (Burn and Rand, 1958; Axelrod, Gordon, Hertting, Kopin and Potter, 1962; Trendelenburg, Muskus, Fleming and Gomez Alonso de la Sierra, 1962a and b; Muscholl, 1966). Tranylcypromine, which is closely related chemically to amphetamine, is also included in this group. However, there are several facets

to tranylcypromine's action and these are described in Chapter 6.

The monoamine oxidase inhibitors listed above and others not mentioned, have been used extensively in the characterization of the enzyme itself and also in the investigation of the biologically active amines, as indicated by the reviews of Davison (1958), Kopin (1964) and Gorkin (1966). The inhibitors which have been mainly employed for the purposes of this thesis are iproniazid, nialamide and tranylcypromine.

The relative insolubility of the particulate mitochondrial monoamine oxidase (Cotzias and Dole, 1951; Barsky, Pacha, Sarkar and Zeller, 1959; Oswald and Strittmatter, 1963) has hindered previous efforts to fully characterize the enzyme and define its properties. It is noted, however, that in addition to mitochondrial monoamine oxidase, appreciable activity, representing a soluble form of the enzyme, was found in the supernatant fluid following ultra-centrifugation of guinea-pig homogenates (Weissbach, Redfield and Udenfriend, 1957). In recent years, intensive efforts have been made to extract the particulate mitochondrial enzyme in a soluble form in order to purify it. Although this has been a difficult task and the results variable, more efficient means of fragmenting the mitochondria and contents with non-ionic detergents (Gorkin, 1963; Nara, Gomes and Yasunobu, 1966), or with ultrasound followed by treatment with detergents (Youdim and Sourkes, 1966; Youdim and

Sandler, 1967), have subsequently been established. These measures, combined with various methods of separation, including ammonium sulphate fractionation and column chromatography, have resulted in a considerable degree of success. For example, Nara, Gomes and Yasunobu (1966) were able to purify beef liver mitochondrial monoamine oxidase by as much as 58 fold and Youdim and Sourkes (1966) obtained purification up to 208 fold over that of the original rat liver homogenate. In addition, Youdim and Sandler (1967) have prepared highly purified monoamine oxidase from human placental mitochondria, obtaining 400 fold purification over that of the original homogenate.

Prior to significant purification of monoamine oxidase, knowledge of the characteristics of the enzyme had been gained by inference from indirect evidence (Blaschko, 1952; Davison, 1958; Belleau and Moran, 1963). However, the availability now of purified soluble enzyme preparations has facilitated direct investigation of these properties. For example, Youdim and Sourkes (1966) showed that the purified enzyme obtained from rat liver homogenates was pale yellow in colour, with a molecular weight of approximately 290,000. The absorption spectrum of the enzyme exhibited maxima at wavelengths of approximately 280 mμ and 410 mμ. Solutions of the enzyme did not fluoresce in the visible or ultraviolet ranges. Ultracentrifugation of a highly purified preparation of monoamine

oxidase showed a single peak with a sedimentation constant of 6.3 and an enzyme preparation was found to contain 0.12% iron and 0.034% copper. The enzyme could be stored in 0.05 M phosphate buffer, pH 7.4 at 4°C with little loss of activity for at least 2 weeks and preparations were stable in the pH range 5.5 to 9.5 but high rates of inactivation occurred outside of this range.

Other characteristics of monoamine oxidase include inhibition by mercaptide-forming reagents (Lagnado and Sourkes, 1956; Barbato and Abood, 1963; Nara, Gomes and Yasunobu, 1966), indicating the importance of sulphydryl groups in its structure. There is evidence suggesting that monoamine oxidase is a flavoprotein (Wiseman-Distler and Sourkes, 1963; Nara, Igaue, Gomes and Yasunobu, 1966) and there have also been indications that copper may be a prosthetic group of the enzyme (Nara, Gomes and Yasunobu, 1966) since known copper chelating agents were found to inhibit the enzyme. There are, however, certain observations which cast doubt on the likelihood of monoamine oxidase being a copper enzyme. For instance, the purified enzyme prepared by the latter group of workers, contained 0.07% cupric copper, whereas the preparation of Youdim and Sourkes (1966), which was of considerably greater purity, paradoxically contained a lower copper concentration of 0.034%. Further negative evidence is provided by the fact that the valency of the (cupric) copper in the enzyme remains unaltered by the

addition of substrate, indicating an apparently static role in the enzyme-substrate interactions involved (Nara, Gomes and Yasunobu, 1966).

As indicated previously in this introduction, it is highly probable that mitochondrial monoamine oxidase is not a single enzyme. The existence of multiple forms of monoamine oxidase has been suspected for many years as it has always displayed great diversity of substrate specificity and inhibitor sensitivity (Bhagvat, Blaschko and Richter, 1939; Alles and Heegaard, 1943; Pratesi and Blaschko, 1959; Hope and Smith, 1960; Maître, 1967). This view has been supported by more recent experiments in which two fractions obtained after separation of rat liver mitochondrial monoamine oxidase displayed differential substrate affinities (Gorkin, 1963). In addition, Youdim and Sandler (1967) reported the separation by polyacrylamide gel electrophoresis of three forms of partially purified monoamine oxidase from rat liver mitochondria and there appeared to be two isoenzymes in a highly purified preparation obtained from human placental mitochondria. Three forms of mitochondrial monoamine oxidase have also been described in tissues of the mouse (Squires, 1968). The latter author noted that the relative proportions of these three forms varied greatly from one organ to another and he suggested that some of the pharmacological effects of the monoamine oxidase inhibitors may depend upon

specific inhibition of single forms of the enzyme. Other studies have included data indicating the presence of four monoamine oxidase isoenzymes in human brain following polyacrylamide gel electrophoresis (Collins, Sandler, Williams and Youdim, 1970).

Evidence favouring multiple forms of monoamine oxidase has also been provided by the fact that certain inhibitors have been found to selectively inhibit limited fractions of the total enzyme content of some preparations. For example, Squires and Buus Lassen (1968) noted that mouse brain monoamine oxidase was only partially inhibited by NSD 2023, both *in vivo* and *in vitro*, using tryptamine as substrate. They suggested that the tryptamine was deaminated by two or more different forms of monoamine oxidase, not all of which were inhibited by NSD 2023. In addition, the experiments of Parmar, Poulose and Bhargava (1967) have demonstrated that the administration to dogs of a monoamine oxidase inhibitor such as pargyline resulted in variations in the degree of inhibition in different anatomical areas of the brain.

The role of monoamine oxidase in the metabolism of the catecholamines under physiological conditions still remains to be fully clarified. In recent years, much convincing indirect biochemical evidence (Kopin and Gordon, 1962a and b, 1963; Kopin, 1964; Snyder, Fischer and Axelrod, 1965) has accumulated, indicating that monoamine oxidase is present within noradrenergic nerves and nerve

terminals. Additional indirect supportive evidence has been provided by means of noradrenaline fluorescence histochemistry (Hamberger, Malmfors, Norberg and Sachs, 1964; Malmfors, 1965). There is also biochemical and histochemical evidence that mitochondrial monoamine oxidase probably undergoes proximo-distal transport from the nerve cell bodies to the terminal axons (Dahlström, Jonason and Norberg, 1969). These latter workers ligated the sciatic nerves of the rat and, at various intervals of time, they estimated the monoamine oxidase content of the nerve sections in the regions above and below the site of constriction. They found that there was an accumulation of the enzyme above the site of constriction which became maximal 7 days after ligation. After lumbar sympathectomy, the enzyme activity in unligated sciatic nerves was not changed significantly, indicating that the noradrenergic axons in normal unligated nerves probably only contained a very small proportion of the total monoamine oxidase content of the sciatic nerve. However, following ligation in sympathectomized sciatic nerves, the increase in enzyme activity above the constricted section was significantly lower after 7 days than that occurring in ligated normal nerves and the maximal accumulation of activity was not seen until later (14 days after ligation). Both maximum levels were, however, of the same order of magnitude. The earlier peak of activity in the normal nerve 7 days after ligation was attributed to accumulation of the enzyme in the

noradrenergic axons and it was suggested that monoamine oxidase in the noradrenergic axons might be transported proximo-distally at a faster rate than in the myelinated axons of the sciatic nerve. In the light of the previously-discussed evidence favouring a mitochondrial localization of the enzyme, it would seem that the mitochondria (containing monoamine oxidase) undergo proximo-distal transport within the noradrenergic neurone.

For a long time, monoamine (amine) oxidase was thought to be the agent primarily responsible for the enzymatic degradation of the catecholamines *in vivo* and it was suggested that it had a function at sympathetic nerve terminals analogous to that of acetylcholinesterase at cholinergic nerve terminals (Burn and Robinson, 1952; Burn, 1953). This hypothesis was largely based on evidence suggesting that the monoamine oxidase content (determined manometrically) of nictitating membrane, iris and foreleg arteries of the cat fell to a variable extent following sympathetic denervation of these tissues (Burn and Robinson, 1952). However, further investigation of these changes in the iris and foreleg arteries of the cat, performed in the same laboratory (Burn, Philpot and Trendelenburg, 1954), failed to confirm the previous findings. In addition, the manometric studies of Armin, Grant, Thompson and Tickner (1953) indicated that the monoamine oxidase content of the central artery of the rabbit ear remained unchanged following sympathetic denervation.

More recent investigations, however, have continued to produce controversial results. For example, some biochemical studies (Snyder, Fischer and Axelrod, 1965; Almgren, Andén, Jonason, Norberg and Olson, 1966) have revealed diminution of monoamine oxidase activity in sympathetically-denervated tissues, whereas other biochemical and histochemical studies (Strömblad, 1956; Fujiwara, Tanaka, Hikosaka and Okegawa, 1966) have failed to demonstrate such changes. The explanation of these variable findings may simply be that the monoamine oxidase content of sympathetic nerves is very small compared with that in other cells, so that the results of sympathetic denervation have depended upon the particular experimental technique and the tissue employed. In addition, the histochemical studies of Koelle and Valk, Jr (1954) in mammalian tissues, indicated that there was no selective association of monoamine oxidase with the noradrenergic nerves in contrast to the specific localization of acetylcholinesterase at cholinergic synapses (Koelle, 1951).

The application of more sophisticated techniques of examining sympathetic nerves and noradrenergically-innervated tissues has meant that the various pharmacological and physiological findings can be interpreted in the light of accurate anatomical and histochemical evidence. The development of the fluorescence histochemical method (Falck, 1962) enabled the localization of

noradrenaline in noradrenergic tissue by means of specific fluorescence. Reports have indicated (Falck, 1962; Norberg and Hamberger, 1964; Malmfors, 1965) that the postganglionic smooth (main and preterminal) axons exhibited only faint fluorescence. However, the terminal fibres, forming an autonomic ground plexus which was demonstrated previously by Hillarp (1959), using conventional histochemical techniques, showed strong fluorescence in the form of abundant small enlargements or varicosities. These varicosities were irregularly spaced along the terminal fibres; their thickness varied from 0.4 to 2 μ (generally from 0.8 to 1.2 μ) and the length between varicosities from 0.5 to 3 μ (Norberg and Hamberger, 1964). There were approximately 15 to 30 varicosities per 100 μ length of fibre. Studies involving electron microscopy indicated that mitochondria and numerous vesicles were situated within the nerve terminals (varicosities) and a variable number of the vesicles also contained more densely-staining cores (Lever and Esterhuizen, 1961; Richardson, 1962, 1964; Lever, Graham, Irvine and Chick, 1965; Grillo, 1966). Lesser numbers of mitochondria and vesicles were also seen in the axoplasm of the non-terminal axons. It is possible that the granulated vesicles represent a catecholamine storage form (Richardson, 1962, 1964; Grillo, 1966; Van Orden III, Bloom, Barrnett and Giarman, 1966).

Centrifugation of tissue homogenates has provided

considerable biochemical evidence that noradrenaline is located within subcellular vesicles (storage particles) in sympathetic nerve fibres and nerve terminals. Such tissues have included bovine splenic nerve (Euler and Hillarp, 1956), rabbit heart (Euler and Lishajko, 1965) and rat heart (Potter and Axelrod, 1962; Michaelson, Richardson, Snyder and Titus, 1964; Snyder, Michaelson and Musacchio, 1964). However, in the more highly purified preparations (Euler and Lishajko, 1965; Michaelson, Richardson, Snyder and Titus, 1964; Snyder, Michaelson and Musacchio, 1964) considerable proportions of the total noradrenaline content have been noted in the soluble fractions, and it is not clear whether this is largely due to the technical procedures involved, or whether a proportion of the noradrenaline in the fibres and terminals is located outside the storage granules. Fluorescence histochemical data, however, have provided indirect support for the concept that most of the noradrenaline is contained within the storage granules in the normal situation and is thus protected from destruction by intraneuronal monoamine oxidase (Hamberger, Malmfors, Norberg and Sachs, 1964; Malmfors, 1965; Norberg, 1967). There is also good fluorescence histochemical evidence that these storage granules are formed in the cell bodies of the noradrenergic postganglionic axons and are transported, along with their enzyme machinery for synthesizing noradrenaline, down to the nerve terminals (Dahlström and

Fuxe, 1964 and b; Dahlström, 1965; Dahlström and Häggendale, 1966, 1967). Noradrenaline is thus synthesized along the whole neurone, but especially in the terminals where storage occurs.

Apart from monoamine oxidase which functions intraneuronally, with the formation of deaminated metabolites (Kopin and Gordon, 1962a and b, 1963; Kopin, 1964), another enzyme of considerable importance in the metabolic inactivation of noradrenaline *in vivo* is catechol-O-methyl transferase. This enzyme, discovered by Axelrod (1957), O-methylates noradrenaline and other catecholamines, with the formation of O-methylated derivatives. It is highly soluble and is distributed widely in mammalian tissues, being particularly abundant in liver and kidney and also present in such organs and tissues as spleen, heart, salivary glands and the central nervous system (Axelrod and Tomchick, 1958; Axelrod, Albers and Clemente, 1959). The enzyme is probably not present in sympathetic nerves (Potter, Cooper, Willman and Wolfe, 1965; Iversen, Glowinski and Axelrod, 1966). Since the establishment of the metabolic pathways for the metabolism of the catecholamines (Axelrod, 1959; Kopin, Axelrod and Gordon, 1961; LaBrosse, Axelrod, Kopin and Kety, 1961; Whitby, Axelrod and Weil-Malherbe, 1961; Kopin and Gordon, 1962a and b), it is now generally accepted that circulating catecholamines are metabolized by O-methylation. In the catalytic reactions involved, catechol-O-methyl transferase requires S-adenosyl-l-

methionine as a methyl donor (Axelrod, 1957) with Mg^{++} or other divalent cations such as Co^{++}, Mn^{++}, Zn^{++}, Fe^{++} or Ni^{++}, for activation (Axelrod and Tomchick, 1958; Senoh, Tokuyama and Witkop, 1962). Competitive inhibition has been achieved with various agents including pyrogallol (Axelrod and Laroche, 1959), catechol flavenoids (Axelrod and Tomchick, 1959) and dopacetamide (Carlsson, Lindqvist, Fila-Hromadko and Corrodi, 1962).

The concept of storage pools or separate compartments of noradrenaline in postganglionic sympathetic nerve terminals has arisen in recent years as it has become clear that the noradrenaline in these regions is not in the form of a single homogeneous entity. This supposition is a logical one as long as it is realized that such pools are not necessarily separated by physical barriers and are more correctly interpreted on kinetic grounds. It has been shown, though, as indicated previously in this introduction, that most of the noradrenaline is bound within storage granules and the actual quantity of noradrenaline situated outside of the granules, in the axoplasm of the nerve fibre, has not been determined satisfactorily. Any free sympathomimetic amine in the axoplasm is probably continuously being deaminated by intraneuronal monoamine oxidase.

Evidence suggesting more than one pool of noradrenaline in sympathetically-innervated tissues has been provided by several groups of workers, employing different methods. Such studies have

included the application of drugs such as the indirectly-acting amine, tyramine (Burn and Rand, 1958) and also reserpine which depletes tissues of noradrenaline by interfering with the retention of this amine in the sympathetic intraneuronal storage granules (Kopin and Gordon, 1962b; Iversen, Glowinski and Axelrod, 1965; Glowinski, Iversen and Axelrod, 1966). The results obtained have suggested the existence of a small pool of labile "available" noradrenaline which is preferentially released by tyramine and which can be refilled from a second stable pool which can be depleted by reserpine but is not immediately available for tyramine release (Trendelenburg, 1961; Crout, Muskus and Trendelenburg, 1962; Potter, Axelrod and Kopin, 1962; Potter and Axelrod, 1963b).

The rate of reserpine-induced noradrenaline depletion from sympathetic nerves in various tissues has been shown to be dependent on impulse flow in such nerves. This is based on evidence that sympathetic decentralization, acute postganglionic denervation or ganglion blocking drugs retarded the rate of disappearance of noradrenaline from sympathetic nerves in various tissues after reserpine treatment (Weiner, Perkins and Sidman, 1962; Hertting, Potter and Axelrod, 1962; Benmiloud and Euler, 1963). Reserpine has been shown to fairly rapidly deplete the noradrenaline stores in decentralized skeletal muscle of the cat; however, a small proportion of the noradrenaline (15% of the total) remained relatively

resistant to reserpine depletion (Sedvall and Thorson, 1963; Sedvall, 1964). This latter portion was depleted by electrical stimulation of the decentralized sympathetic nerve supply (Sedvall and Thorson, 1963). Sedvall (1964) suggested that these results implied the existence of two different pools of noradrenaline in skeletal muscle of the cat; the larger compartment, containing about 85% of the total noradrenaline, was rapidly depleted by reserpine, whereas the remaining 15% was stored in a reserpine-resistant pool which could be rapidly released during impulse activity. Subsequent studies, using ^{3}H-noradrenaline in the rat (Fischer and Kopin, 1964), have confirmed the existence of a small reserpine-resistant intraneuronal store of noradrenaline which could be released by nerve stimulation and these results also suggested that this pool was unaffected by the action of tyramine. In addition, Fischer, Kopin and Axelrod (1965) provided evidence in the chronically-denervated rat salivary gland that there was also an extraneuronal pool of noradrenaline which was resistant to the action of reserpine.

Some of the released noradrenaline is O-methylated and inactivated by catechol-O-methyl transferase in the region of the synapse and some may diffuse from this area and be removed by the circulation. However, it has been widely accepted that the predominant mechanism terminating the action of both endogenously-

released noradrenaline and exogenous noradrenaline is uptake by the nerve terminal (Whitby, Axelrod and Weil-Malherbe, 1961; Hertting, Axelrod, Kopin and Whitby, 1961; Kopin, 1964; Malmfors, 1965). It has been shown (Wakade and Furchgott, 1968) that energy is necessary both for the primary uptake process across the axonal membrane and also for the subsequent incorporation of the noradrenaline in the intraneuronal storage granules.

More recently, Kalsner and Nickerson (1969), working with helically-cut strips of rabbit aorta, placed much less emphasis on the uptake mechanism as a means of inactivating noradrenaline. Their results suggested that enzymatic processes made a considerably more important contribution to the inactivation of low (physiological) concentrations of noradrenaline and that these processes appeared to account for almost all of the inactivation of high noradrenaline concentrations. Inhibition of catechol-O-methyl transferase showed that O-methylation was the major enzymatic mechanism for the inactivation of low noradrenaline concentrations in these experiments. Furthermore, inhibition of monoamine oxidase alone by iproniazid was found to have a negligible effect on noradrenaline inactivation if catechol-O-methyl transferase activity was unimpaired.

These authors postulated that catechol-O-methyl transferase and monoamine oxidase functioned in "series", and they suggested that this behaviour might be explained on the basis that whereas

monoamine oxidase is mainly located in intracellular mitochondria, catechol-O-methyl transferase is predominantly a soluble tissue enzyme. Thus extraneuronal noradrenaline would be exposed to a region of catechol-O-methyl transferase activity before reaching mitochondrial loci of inactivation. Monoamine oxidase did, however, appear to function as an effective alternative mechanism for the inactivation of noradrenaline in the aortic strip when catechol-O-methyl transferase was absent. Indeed, with 100 fold higher concentrations of noradrenaline, monoamine oxidase assumed the more important role of inactivation. Deamination appeared, therefore, to be a major pathway for the inactivation of high concentrations of noradrenaline, compared with its unimportant effects on low concentrations.

Kalsner and Nickerson (1969) explained the marked difference in the contributions of monoamine oxidase to inactivation of low and high noradrenaline concentrations by assuming that the high amine concentration swamped the organized sequential system which inactivates physiological amounts. The net result was a more or less simultaneous presentation of intact noradrenaline to both catechol-O-methyl transferase and monoamine oxidase. Both enzymes therefore revealed their true capacity to separately inactivate noradrenaline.

Thus knowledge concerning the inactivation and metabolic

degradation of noradrenaline and other sympathomimetic amines has advanced rapidly in recent years. Many factors have contributed to this rapid development. The introduction of drugs interfering with monoamine oxidase and amine metabolism, the use of labelled amines and the development of specific histochemical techniques have proved to be major contributions. Of no lesser importance have been the detection and isolation of the amine storage granules and the ability to extract mitochondrial monoamine oxidase in a soluble form, together with electron microscopical visualization of such storage granules and mitochondria. In this dynamic field, however, much of the current information will be out of date in a relatively short period of time. Most of the recent investigations have, in fact, posed as many questions as they have provided answers and the studies reported in this thesis have proved to be no exception to the general rule.

SUMMARY

1. Historical aspects of the fate of amines in the mammalian body have been reviewed and, in particular, the role of monoamine oxidase has been considered in detail.

2. It is apparent that the term "monoamine oxidase" is a collective one involving a number of isoenzymes which vary in their relative proportions in different mammalian tissues and organs. It is possible that certain metabolic effects in such tissues may be related to involvement of specific and separate forms of monoamine oxidase.

3. The physiological action and fate of noradrenaline at the noradrenergic nerve terminal has been studied in the light of the recent advances in the knowledge of noradrenaline uptake mechanisms and the pharmacology of monoamine oxidase and catechol-O-methyl transferase.

4. The concept of noradrenaline storage pools in the noradrenergic neurone and nerve terminal has been discussed with the realization that much remains to be clarified. The consideration of such pools on kinetic grounds has provided a logical means of interpreting some of the dynamic changes in the nerve terminal.

5. Major advances in the knowledge of this dynamic field have

occurred in recent years. However, the greatest importance of the new experimental techniques evolved is that they have opened up new avenues of exploration.

6. The principle function of this thesis has been to further explore some of the complexities of amine inactivation and metabolism involved in the noradrenergic nervous control of vascular tissue.

C H A P T E R 2

GENERAL METHODS

Introduction:

This chapter outlines the basic techniques employed in these studies. The experimental methods described in this chapter are as follows:

Rabbit ear artery perfusion.

Histochemical method for monoamine oxidase.

Histochemical method for sympathetic innervation.

Pretreatment of the experimental animal.

Venous occlusion plethysmography in man.

Modifications of these methods are described separately in later chapters under the heading "Materials and Methods" for these chapters. Further detailed description of equipment used is presented in the Appendix. A list of the drugs employed is also included in the Appendix, together with their origin and a description of the manner in which they were prepared for experimental use.

Rabbit ear artery perfusion:

The male and female semi-lop-eared rabbits used in these studies were from a strain bred at the Central Animal House of the University of Adelaide. The animals varied in weight from 1.5 to

3.5 Kg, with an average weight of 2.4 Kg. They were not fasted prior to experimental use except where ether anaesthesia was employed for the recovery operation of sympathectomy. Non-recovery anaesthesia was induced by means of urethane 7 ml/Kg of a 25% (w/v) solution in normal saline. This solution was injected intraperitoneally, with added increments being given as required. Prior to operation, heparin 1000 units/Kg was given intravenously into an ear vein in order to render the blood non-coagulable.

Isolation and removal of the central ear artery:

The ear (Fig. 2-1) was positioned so as to clearly display the central ear artery, pulsation of which could be seen and felt near the base of the ear. The skin was incised longitudinally adjacent to the central artery and vein and these underlying structures were then exposed by appropriate dissection. The ventral auricular nerve lies parallel to the central vein and although it is predominantly sensory in nature, it contains sympathetic vaso-constrictor fibres (Burn and Rand, 1960).

The central artery was freed from surrounding tissue for a distance extending from its point of emergence from deeper structures near the base of the ear to the first major branch of the artery. The artery was ligated with cotton at its proximal end and also at its distal end, just proximal to the first major branch. In most experiments, the artery was double-cannulated by inserting

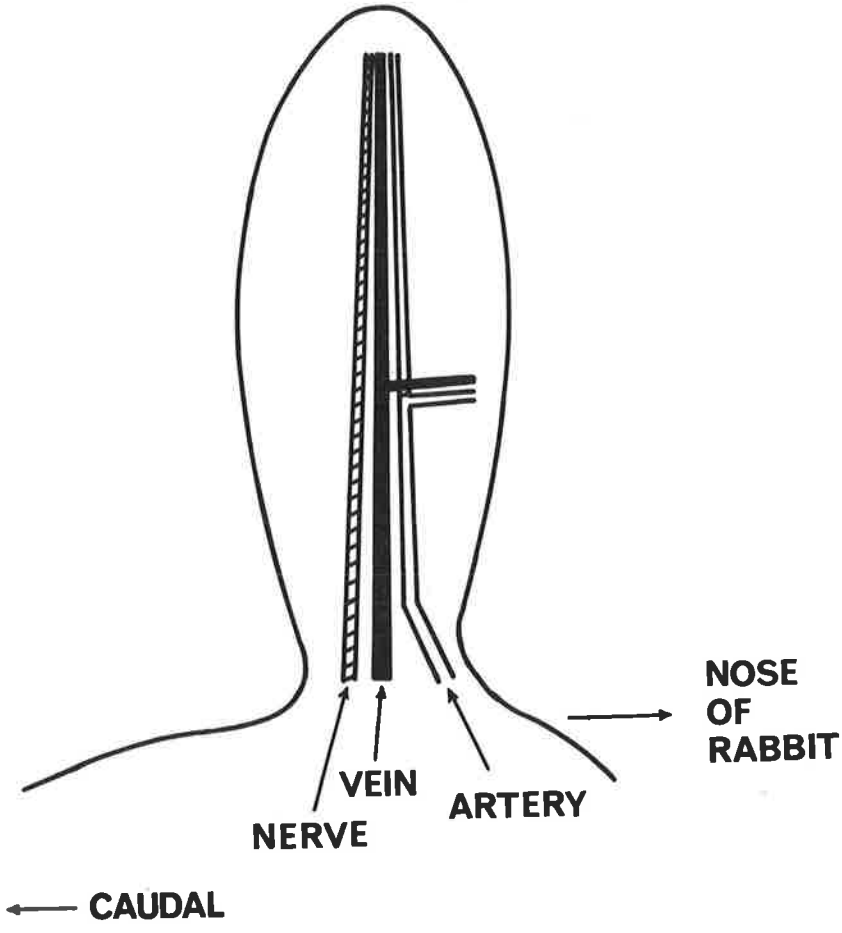


Fig. 2-1 A diagram of the left ear of a semi-lop-eared rabbit illustrating the convex surface of the ear as seen from the mid-line of the skull and showing the relative positions of the ventral (great) auricular nerve, the central vein and the central (main) artery of the ear.

polythene cannulae into the proximal and distal ends of the artery within the cotton ligatures so that there was a 1.5 to 2.0 cm length of artery, without branches, between the tips of the cannulae. In some experimental procedures, the artery was single-cannulated by insertion of the proximal cannula only so that there was a 2 to 3 cm length of artery beyond the tip of the cannula suitable for perfusion.

The single- or double-cannulated vessel was immediately removed and placed into a dish containing warmed bubbled Krebs bicarbonate solution (Appendix page A-2). The vessel was then gently perfused via the proximal cannula with a syringe containing the same solution, in order to remove any debris and also, in the case of the double-cannulated artery, to detect any major leaks. Having ascertained that the cannulation technique was satisfactory, the cannulated artery was immediately transferred to the organ bath of the perfusion apparatus.

Perfusion methods:

The principle of the method of perfusing the isolated central artery of the rabbit ear was that of de la Lande and Rand (1965). In their original technique, a single-cannulated artery which had not been cleaned of adherent tissue, was suspended in Krebs bicarbonate solution in an organ bath of 10 ml volume. The artery was perfused with Krebs bicarbonate solution by means of a

roller pump delivering a constant rate of flow. Changes in vascular resistance produced changes in perfusion pressure which were recorded by a mercury manometer. However, under these experimental conditions, the intraluminal fluid escaped into the extraluminal bathing solution before draining away through the overflow tube of the organ bath.

Three modifications were employed in the present studies. The first was that of de la Lande, Cannell and Waterson (1966), in which the arteries used were largely cleaned of adjacent tissue. Such single-cannulated vessels, perfused with Krebs bicarbonate solution only, were used as normal controls in some of the histochemical experiments. However, in all of the physiological experiments and in nearly all of the histochemical experiments, the arteries were double-cannulated as a second modification, by the method of de la Lande, Cannell and Waterson (1966). By this means, the artery segment was cannulated separately at its proximal and distal ends so that the perfused fluid escaped through the distal cannula and did not come into contact with the extraluminal bathing solution. The third modification was the suspension of the distal cannula from a lever so that the artery remained under a mild tension of 0.4 to 0.7 gm. This prevented the partial occlusion of the artery which sometimes occurred as it lengthened during periods of vasoconstriction.

A diagrammatic representation of the apparatus used for

perfusion of a double-cannulated artery is shown in Fig. 2-2 and that for a single-cannulated artery is identical except that there is no distal cannula and the artery is not under constant tension. Krebs bicarbonate solution was pumped at a constant rate through the artery from a reservoir situated in the heated water bath. A pump in the water bath circulated warmed water through the walls of the double-jacketed organ bath and also through a warming coil in the perfusion circuit. The temperature in the organ bath was maintained at 37°C since the blood vessels of the rabbit ear *in vivo* usually tend to be vasodilated at this temperature (Grant, 1935).

The intraluminal Krebs bicarbonate perfusion solution was bubbled with 5% carbon dioxide in oxygen and a separate gas tube was placed in the Krebs bicarbonate solution surrounding the artery in the organ bath. After leaving the roller pump, the intraluminal solution was passed through the warming coil which maintained it at 37°C before entering the cannulated artery. The effluent from a single-cannulated vessel escaped into the solution in the organ bath and thence from the bath into an overflow tube, whilst that from a double-cannulated artery escaped via the distal cannula. Perfusion pressure in most experiments was measured with a mercury manometer recording on a kymograph and in some experiments, with a pressure transducer recording on a chart recorder. Constriction of the artery was measured as a rise in perfusion pressure.

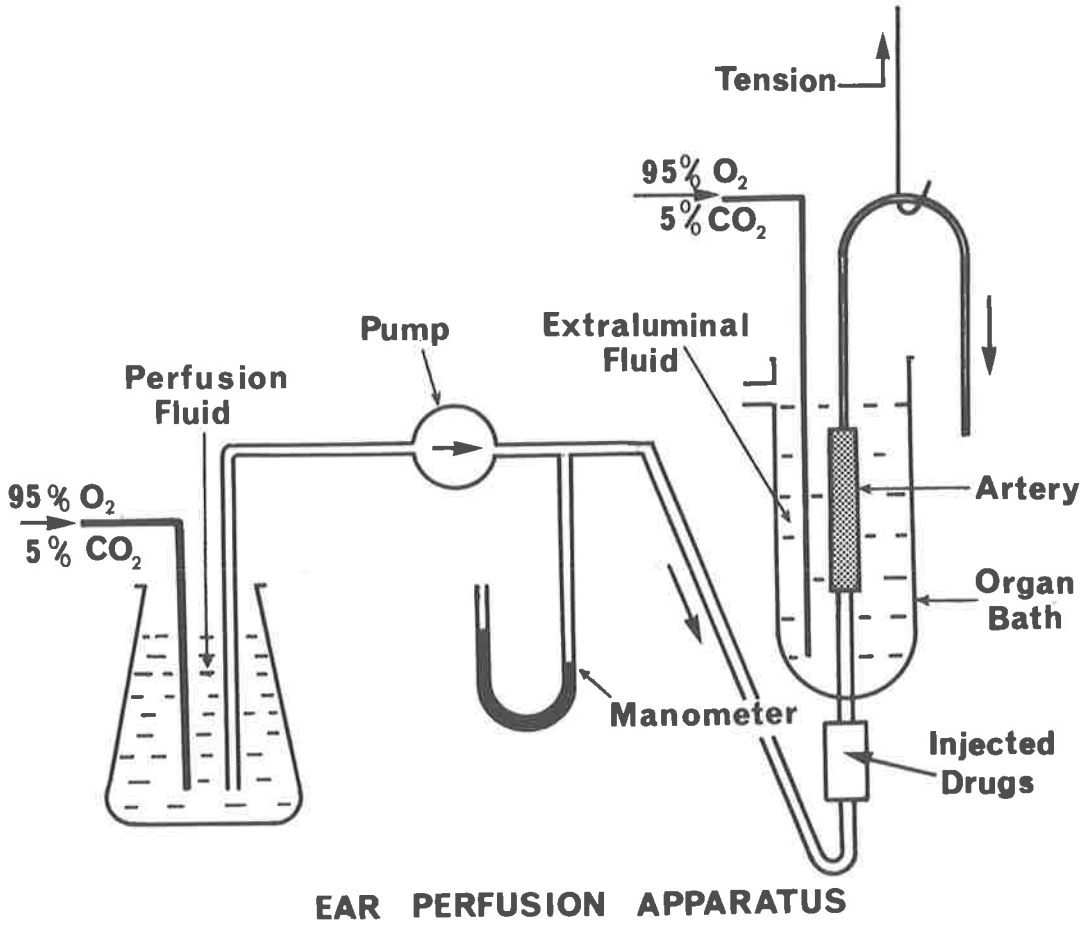


Fig. 2-2 Diagrammatic representation of the apparatus used to perfuse the isolated central artery of the rabbit ear. Double-cannulation method.

The volumes of different organ baths employed were measured accurately and they varied between 10 and 20 ml. The roller pump delivered perfusion fluid at a constant rate; however, the flow rate through the artery varied with diversion of perfusion fluid into the mercury manometer as constriction of the artery increased the pressure within the system. Such changes in rate of flow were slight and in experiments using a pressure transducer to demonstrate changes in perfusion pressure, constrictor responses of the arteries to various drugs were identical in pattern to those seen when a mercury manometer was employed.

Intraluminal flow rates were maintained between 5 and 6 ml/min and these were associated with resting levels of perfusion pressure between 10 and 30 mm of mercury. In double-cannulated arteries, at the beginning of each experiment, the level of the extraluminal fluid in the organ bath was routinely examined for the existence of any leakage between the intraluminal and extraluminal fluids. In addition, at the conclusion of many of the experiments which did not involve histochemical procedures, the artery was perfused with Evans blue dye (10 mg/ml of Krebs bicarbonate solution), with subsequent photometric comparison of control Krebs bicarbonate solution with intraluminal and extraluminal samples of solution removed following a 10 minute period of dye perfusion. Fig. 2-3 represents typical findings in 4 experiments. Approximately one

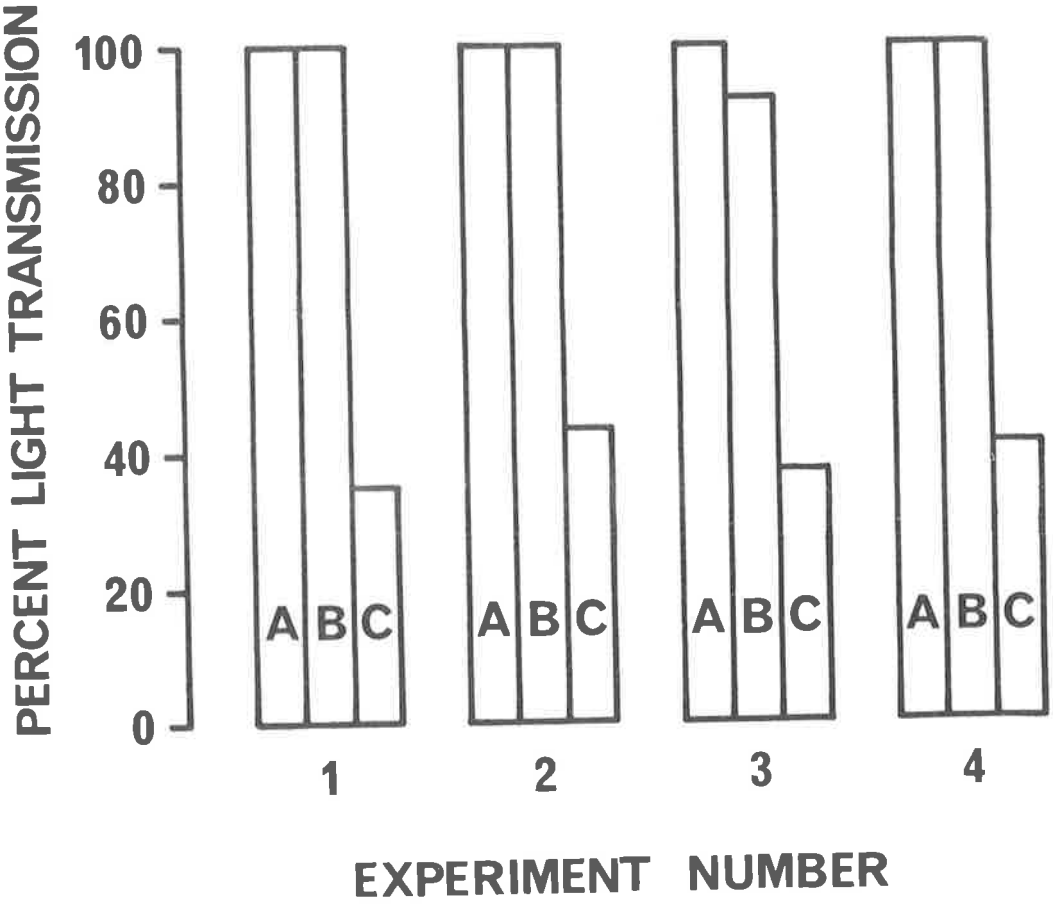
Fig. 2-3 Results of 4 dye perfusion experiments, testing for possible leakage in 4 double-cannulated arteries. The height of each column is a measure of the light transmission, measured photometrically, through the fluid sample.

A = Control Krebs bicarbonate solution.

B = Extraluminal fluid sample, taken following intraluminal dye perfusion for 10 minutes.

C = Intraluminal dye perfusion fluid sample collected via the distal cannula following dye perfusion for 10 minutes.

No leaks were evident in experiments 1, 2 and 4; however, the reduced light transmission in sample B in experiment 3 indicates that this artery had a slight leak.


PERCENT LIGHT TRANSMISSION
100
80
60
40
20
0
A B C
A B C
A B C
A B C
1
2
3
4
EXPERIMENT NUMBER

artery in seven or eight showed evidence of leakage, usually through a small side branch which was not detected during the dissection, and such arteries were discarded.

Drugs were dissolved in 0.9% w/v saline solution and administered intraluminally to the artery either by injection into the perfusion stream via the rubber tubing directly proximal to the organ bath or they were added directly to the reservoir of the intraluminal perfusion solution situated in the heated water bath. Drugs were applied extraluminally to the artery by adding them to the extraluminal fluid bathing the artery in the organ bath. Concentrations of the drugs were recorded as weight/ml in the intraluminal perfusion fluid and in the extraluminal bathing solution, and the drugs were added in volumes of 0.05 to 0.1 ml/100 ml of intraluminal solution or in volumes of 0.05 to 0.2 ml/10 ml of extraluminal solution. Drugs injected into the intraluminal perfusion stream immediately proximal to the artery were administered in volumes ranging between 0.05 and 0.4 ml. Replacement of drug-containing perfusion fluid with drug-free perfusion fluid and successive flushing of the extraluminal bathing solution with aliquots of fresh perfusion fluid, respectively, were the means by which drugs were removed from the intraluminal and extraluminal fluids.

Previous experience had shown (de la Lande and Waterson, 1968a) that the sensitivity of the artery to drugs, especially to

noradrenaline, changed considerably during the first hour of perfusion. Accordingly, experiments were routinely commenced after the artery had been perfused with Krebs bicarbonate solution for 1 hour. However, when an artery had been perfused initially with a monoamine oxidase inhibitor such as iproniazid or nialamide for 1 hour, the drug was then washed out and the artery perfused with Krebs bicarbonate solution for 10 minutes prior to commencement of the experimental procedure.

Stimulation of sympathetic nerves in the artery wall:

Field stimulation was applied by means of a Grass stimulator which delivered pulses to the artery via two electrodes which were positioned in the organ bath so that one was below the artery and the other was above the artery on the opposite side. Pulses were usually of 1 millisecond duration with a frequency of 2 to 10 per second. For single responses, the train of impulses was applied for 10 seconds routinely. Voltage was increased until it was supramaximal and this varied between 20 and 100 volts.

Measurement of vascular sensitivity changes:

The constrictor response to a drug such as noradrenaline was recorded as the maximum sustained rise in perfusion pressure which occurred during its application. Such responses (Fig. 2-4) were recorded in duplicate at two concentration levels of the drug and, from these data, concentration-response curves (for convenience

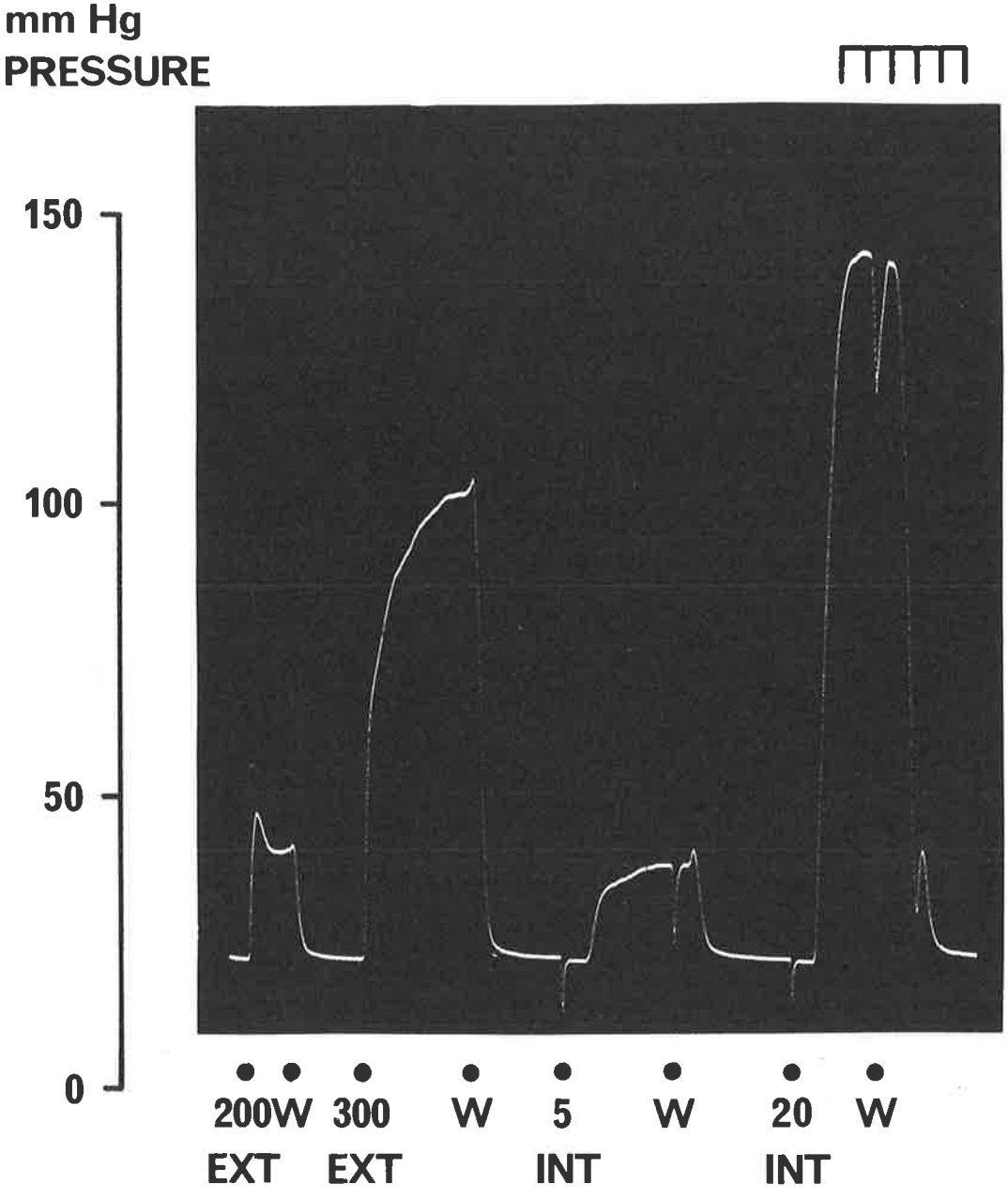


Fig. 2-4 Constrictor responses of a control (untreated) ear artery to extraluminal (EXT) and intraluminal (INT) noradrenaline in the doses (ng/ml) as indicated by the numerals. W refers to noradrenaline washout.

Time scale: minutes

called dose-response curves) were established as described for noradrenaline by de la Lande, Cannell and Waterson (1966), and de la Lande, Frewin and Waterson (1967). Changes in sensitivity of the artery to the drug were established by the ratio of the drug concentrations producing constrictor responses of equal magnitude. This ratio was referred to as the sensitivity ratio and its interpretation depends upon the manner in which it is employed.

The change in sensitivity to noradrenaline or histamine produced by the application of a second drug such as nialamide or tranylcypromine, was estimated by means of the sensitivity ratio:

$$\frac{\text{dose of noradrenaline or histamine before drug application}}{\text{dose of noradrenaline or histamine after drug application}}$$

producing responses of equal magnitude. For example, if the response to a 50 ng/ml dose of noradrenaline before the application of nialamide equalled that to a 5 ng/ml dose of noradrenaline after nialamide application, the value of the sensitivity ratio was calculated as 10. The sensitivity ratio in this context is the inverse of the dose ratio which is usually applied to the change in sensitivity produced by an antagonist and which is the

$$\frac{\text{dose of stimulant after application of the antagonist}}{\text{dose of stimulant before application of the antagonist}}$$

producing responses of equal magnitude. The advantage of employing the sensitivity ratio instead of the dose ratio as an index of change in sensitivity, is that the former permits a ratio of greater

than one to represent a gain in sensitivity, whereas it is customary to associate a dose ratio of greater than one with antagonism.

With a drug such as tyramine, determination of vascular sensitivity change was complicated by the propensity for tyramine sensitivity to decline during the experimental procedure. Although changes in sensitivity were still expressed by means of sensitivity ratios, several different experimental methods were employed in order to estimate equipotent intraluminal and extraluminal tyramine doses in the presence and absence of monoamine oxidase inhibition. In these experiments, it was also important to estimate the differences between intraluminal and extraluminal tyramine potencies in control (untreated) and monoamine oxidase-inhibited arteries. These methods and the sensitivity ratios used are fully described in Chapter 4.

Histochemical method for monoamine oxidase:

The tetrazolium method of Glenner, Burtner and Brown, Jr (1957) was used in the studies relating to the distribution of monoamine oxidase in the artery wall. The principle of this method is that monoamine oxidase in the tissue section oxidizes a substrate, tryptamine hydrochloride, to indolyl-3-acetaldehyde, which in turn reduces a nitro-blue tetrazolium dye to an intense blue-staining precipitate. This blue precipitate which is present in both diffuse and particulate form, indicates the position of monoamine oxidase in the tissue under examination.

Two modifications of the original technique were employed. Firstly, excised ear arteries which had been perfused as described previously, were routinely incubated for 2 hours instead of 30 to 45 minutes as in the original method. The longer incubation period for these vessels was selected after preliminary experiments had indicated that staining was thus more intense and clearly defined. All other tissue sections were routinely incubated for 45 minutes, although longer periods of time (up to 2 hours) were often also used in conjunction, in attempts to better delineate certain types of tissue, for example, nerve fibres. Incubation periods longer than 45 minutes were also employed by Härkönen (1964) and Fujiwara, Tanaka, Hikosaka and Okegawa (1966). Secondly, sections were mounted in glycerine jelly instead of dehydrating, cleaning and mounting in Permount as suggested by the original authors. Pearse (1960) proposed the former procedure as the latter method removes certain purple and red colours which may also indicate monoamine oxidase activity.

The histochemical procedure in detail was as follows:- The rabbit tissues examined were the excised central ear artery, the whole ear containing the vessels and nerves *in situ* and the kidney. Fresh frozen cryostat sections (10 to 20 μ in thickness) were cut at a temperature of -25°C, mounted on glass slides and air-dried for 10 to 15 minutes. The sections were then incubated at 37°C for either

45 minutes or 2 hours routinely in a medium consisting of tryptamine hydrochloride 25 mg, sodium sulphate 4 mg, nitro-blue tetrazolium 5 mg, 5 ml of 0.1 M phosphate buffer (pH 7.6) and 15 ml of distilled water. Following incubation, the slides were washed in running water for 2 minutes, the sections were fixed in 10% neutral formalin for 24 hours and then mounted in glycerine jelly.

Instead of the medium containing nitro-blue tetrazolium, sections of each tissue were also incubated in a medium containing tetranitro-blue tetrazolium. This latter tetrazole is more easily reduced than nitro-blue tetrazolium and accordingly the transfer of electrons from the indolyl-3-acetaldehyde is more efficient (Pearse, 1963). Theoretically, accuracy of localization depends upon the mechanism of the reaction and upon the efficiency of the capture stage by which the insoluble formazan precipitate is produced. The primary reaction product (indolyl-3-acetaldehyde) produces a rapid and direct reduction of the respective tetrazole and there is no evidence to suggest the participation of flavoprotein enzymes (diaphorases) in the reaction (Glenner, Weissbach and Redfield, 1960).

Control tissues:

In order to distinguish between enzymatic and artifactual staining, control tissues were processed with each group of tissue sections. Kidney tissue was employed routinely since monoamine oxidase is present in high concentration and its distribution is

well-documented (Koelle and Valk, Jr, 1954; Glenner, Burtner and Brown, Jr, 1957; Graham, Jr, and Karnovsky, 1965). Kidney sections which had been incubated in the absence of tryptamine hydrochloride were also utilized.

Histochemical method for sympathetic innervation:

The technique for showing the location and distribution of the sympathetic nerve terminals in the central ear artery of the rabbit under the various experimental conditions employed in these studies was based on the method developed by Falck (1962) as modified by Waterson and Smale (1967). The principle of Falck's original method involved freeze drying of the tissue followed by exposure to formaldehyde gas to convert the catecholamines present to isoquinolones. The treated tissue was then embedded in paraffin wax and sections were cut and examined by fluorescence microscopy. The two modifications of this method, described by Waterson and Smale (1967) and used in the present studies, were as follows:

1. A mixture of acetone and dry ice was used to rapidly freeze the tissues (Fujiwara, Tanaka, Honjo and Okegawa, 1965) instead of isopentane cooled in liquid nitrogen as employed by Falck (1962).

2. When the process of freeze drying had been completed, the specimens were transferred directly to a glass jar containing paraformaldehyde, whereas in Falck's method, the specimens were placed

first into a perspex box containing a dessicant (phosphorus pentoxide) in order to prevent the uptake of moisture during the transfer process.

The following is a detailed description of the histochemical technique employed in the present studies. A fresh artery segment at least 1 cm long was quickly plunged into the acetone and dry ice mixture in a glass container so that the artery was rapidly frozen. Usually several artery segments were to be examined at the same time and after each one was frozen, it was placed into a numbered glass tube situated in dry ice in an insulated metal container. The arteries were quickly transferred to the freeze drying apparatus (Thermovac, Model FD/3) which had been previously cooled to at least $-40^{\circ}C$ so that the specimens did not thaw. The specimens were freeze dried for 16 to 20 hours at temperatures ranging from $-50^{\circ}C$ to $-40^{\circ}C$ and at pressures of 15 to 50 microns of mercury. Following this procedure, the specimens were removed and placed into a large glass jar containing 5 grams of paraformaldehyde powder which had been stored over 34% v/v sulphuric acid at a relative humidity of 70% for at least 7 days (Appendix, page A-4). This standardization of the paraformaldehyde powder for water content (Hamberger, Malmfors and Sachs, 1965) was essential if significant fluorescence due to formaldehyde treatment was to be seen. The glass jar was sealed and placed into an oven which had been preheated to $80^{\circ}C$.

Heat incubation for 1 hour was sufficient for the development of the noradrenaline fluophore (Falck and Owman, 1965). After this time, the artery specimens were removed and vacuum infiltrated for 30 minutes with paraffin wax at a temperature of 60°C. They were then embedded in paraffin wax and blocks were trimmed to the required size. Subsequently tissue sections were cut at a thickness of 7 microns and mounted in an Entallan (Merck) and xylol mixture. The tissue sections were examined by fluorescence microscopy and photographed as described in the Appendix, page A-4.

The appearance of the rabbit ear artery after treatment by the paraformaldehyde method outlined above has been fully described by Waterson and Smale (1967), and de la Lande, Frewin and Waterson (1967). An example of such an artery is illustrated in Fig. 2-5. Two circular regions of fluorescence are apparent: an inner one at the site of the intima and an outer one at the medial-adventitial border of the vessel. The intimal fluorescence is green-yellow in colour and this represents the non-specific autofluorescence which has been reported in blood vessels previously (Carlsson, Falck and Hillarp, 1962; Fuxe and Sedvall, 1965). The outer ring of noradrenergic fluorescence appears as light green or green-yellow fluorescent structures located in the adventitia adjacent to the outer border of the smooth muscle layer of the media.

In order to show that the formaldehyde method was specific

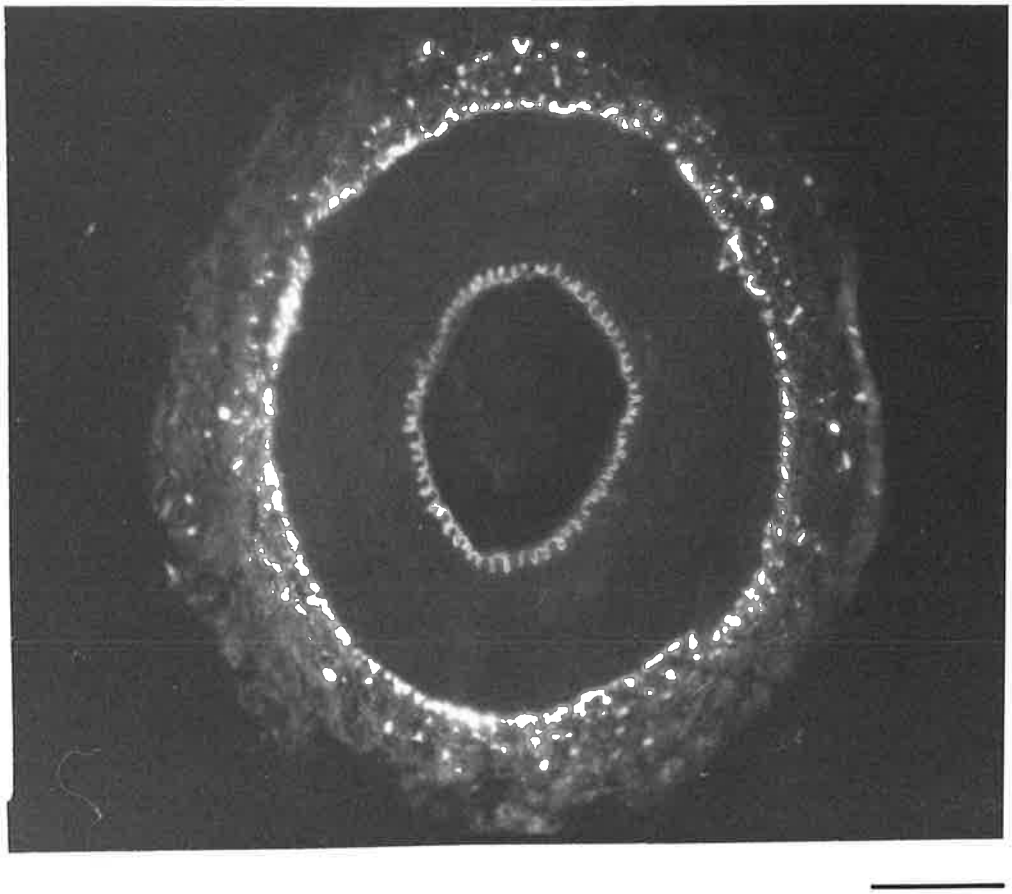


Fig. 2-5 Transverse section of a rabbit ear artery examined by the noradrenaline fluorescence technique, showing noradrenergic fluorescence at the medial-adventitial border of the vessel and intimal autofluorescence.

Section thickness: 7 microns

Formaldehyde treatment: 1 hour

Scale: 100 microns

for catecholamines, the following specificity tests were employed.

1. Sections of formaldehyde-treated arteries were exposed to water on the microscope slides prior to mounting, since water removes specific catecholamine fluorescence (Falck and Owman, 1965). The ring of fluorescent structures at the medial-adventitial border of each artery was no longer apparent, but the autofluorescence in the intima remained unchanged.

2. Artery segments treated by the usual process, except that paraformaldehyde was omitted during the stage of heat incubation, did not show fluorescence at the medial-adventitial border, although intimal autofluorescence was still evident.

3. The borohydride test for specificity of catecholamine fluorescence was applied to artery sections (Corrodi, Hillarp and Jonsson, 1964). In 3 experiments, sections of formaldehyde-treated arteries were washed in 0.1% sodium borohydride dissolved in 90% isopropanol. Examination by fluorescence microscopy confirmed that the borohydride solution had eliminated the outer ring of noradrenergic fluorescence which was later restored by further exposure to formaldehyde vapour.

4. Examination of arteries removed from nine of the 13 rabbits which had been pretreated with reserpine 24 hours previously, as described later in this chapter, did not show any fluorescence at the medial-adventitial border, whereas the intimal autofluorescence

was unaffected. Reserpine has been shown to interfere with the retention of noradrenaline in the noradrenergic intraneuronal storage granules (Kopin and Gordon, 1962b; Iversen, Glowinski and Axelrod, 1965; Glowinski, Iversen and Axelrod, 1966). The net effect was the disappearance of noradrenaline from the reserpine-treated tissue.

5. As described later in this chapter, chronic sympathetic denervation of one ear in each of 17 rabbits by the technique of de la Lande and Rand (1965) caused the disappearance of fluorescence at the medial-adventitial border of the denervated arteries, as illustrated by Fig. 2-6. These results confirmed the earlier findings of de la Lande, Frewin and Waterson (1967) in the rabbit ear artery. Chronic postganglionic sympathetic denervation had the effect of destroying the noradrenergic storage structures resulting in loss of noradrenaline from the tissue.

In addition to these specificity tests, de la Lande and Waterson (1968b) have shown that treatment of the ear artery with Evans Blue dye (50 µg/ml of Krebs bicarbonate solution) prior to formaldehyde treatment produced a marked change in the intimal autofluorescence which now had a dull red appearance. It could thus be easily distinguished from the fluorescence at the medial-adventitial border which remained unchanged. de la Lande and Head (1967) also analyzed segments of rabbit ear arteries for their

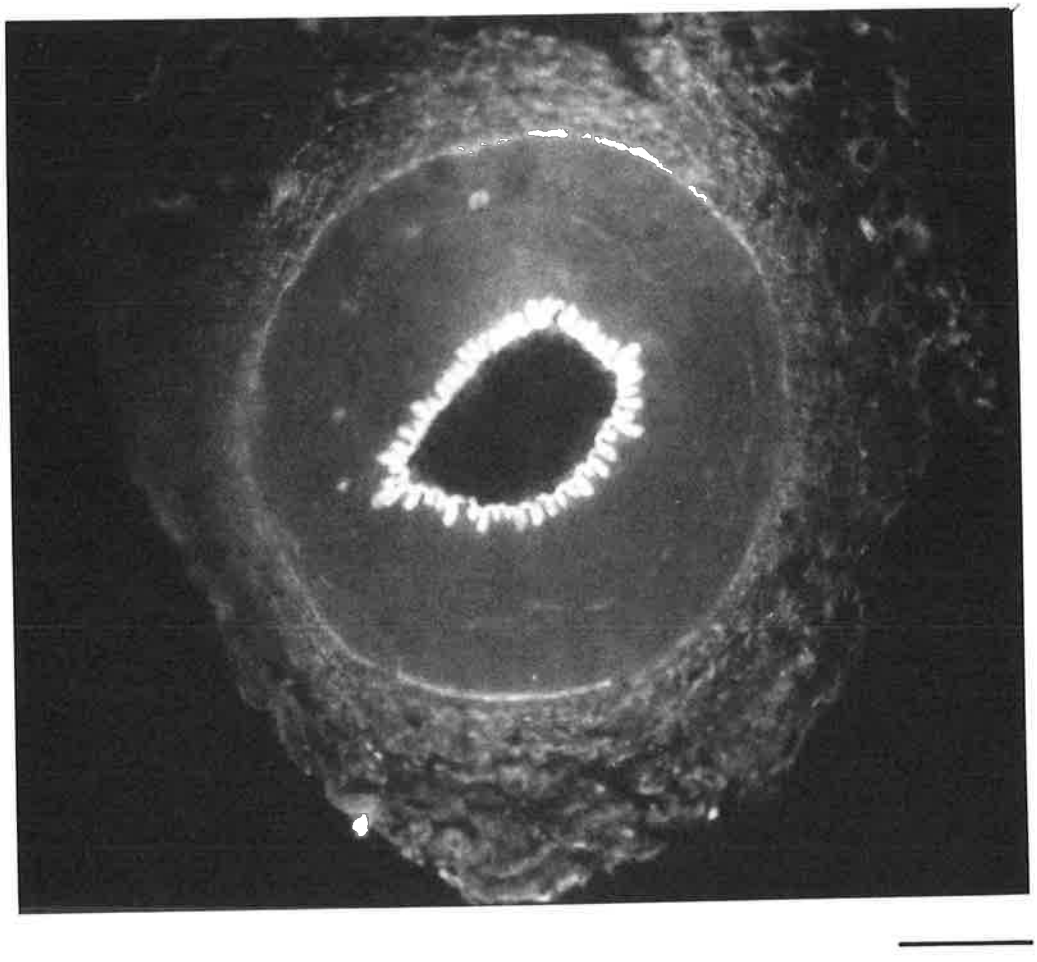


Fig. 2-6 Transverse section of a rabbit ear artery following sympathetic denervation 21 days previously. Examination by the noradrenaline fluorescence technique shows absence of noradrenergic fluorescence at the medial-adventitial border of the vessel; however, the intimal autofluorescence is still present.

Section thickness: 7 microns

Formaldehyde treatment: 1 hour

Scale: 100 microns

catecholamine content by the fluorimetric method of Euler and Lishajko (1959). They concluded that noradrenaline was the predominant amine in the ear artery of the rabbit.

In view of the above findings, there is thus considerable evidence that the fluorescence seen at the medial-adventitial border of the rabbit ear artery is due to noradrenaline contained in the sympathetic nerve terminals.

Pretreatment of the experimental animal:

Rabbits were pretreated in some experiments in order to inhibit the action of monoamine oxidase in the blood vessels of the ear and also to modify the function of the noradrenergic storage sites (the sympathetic nerve terminals) in these blood vessels. By this means, further information could be gained concerning the relationship between the actions of monoamine oxidase, the nerve terminals and various drugs applied to the ear artery. Monoamine oxidase was inhibited by the *in vivo* application of one of the monoamine oxidase inhibitors, either iproniazid or nialamide, and the function of the sympathetic nerves and nerve terminals was altered either by sympathetic denervation or by pretreatment of the rabbit with reserpine.

Inhibition of monoamine oxidase in vivo:

This was achieved by the intraperitoneal administration of iproniazid (200 mg/Kg body weight) 24 hours prior to the commencement

of some of the experimental procedures described in Chapters 3 and 4.

Sympathetic denervation:

For the purposes of a number of experiments described in Chapters 3, 5 and 6, the central artery of the left ear was denervated in each of 26 rabbits by removal of the respective superior cervical ganglion. The sterile surgical technique for sympathetic denervation was that employed by de la Lande and Rand (1965). After premedication with 2.5 mg/Kg of atropine sulphate intramuscularly 30 minutes previously, anaesthesia was induced and maintained with ether. The skin of the neck was clipped and prepared with a solution of 10% cetrimide in alcohol and the immediate area of the proposed incision was infiltrated with 1 ml of 2% lignocaine.

The superior cervical ganglion in the rabbit lies at the level of the angle of the mandible and a midline incision was made in the neck of the animal so that it included this level. Blunt dissection exposed the trachea, the left carotid artery and the left cervical sympathetic nerve. The ganglion was held with fine forceps and the pre- and postganglionic nerve fibres were cleared for a distance of a centimetre or so, then the ganglion, together with these attached fibres, was excised. After cessation of any bleeding, the neck wound was closed in layers and the suture line was sprayed with nobecutane. Intramuscular procaine penicillin 100,000 units/Kg was then administered to all animals.

The effectiveness of the superior cervical ganglionectomy was evidenced by constriction of the pupil and by early vasodilatation of the ear blood vessels on the side of the operation. Segments were taken for monoamine oxidase histochemical examination from both the denervated and opposite control ear arteries of twelve animals, 9 to 24 days after the operation. Similar segments were removed from two other animals 30 days and 60 days, respectively, after ganglionectomy. Lengths of these ear arteries were also double-cannulated and perfused by the method described previously in this chapter and the effectiveness of denervation was further tested by the absence of a constrictor response to a degree of field stimulation which produced massive constriction of the opposite control ear artery. The conditions of electrical stimulation were as described previously in this chapter. Occasionally a very slight response to electrical stimulation occurred in the denervated artery but this was always of very much less magnitude than in the corresponding control artery. The arteries from seventeen of the 26 rabbits (6 to 30 days after sympathectomy) were also examined by fluorescence microscopy following treatment with formaldehyde. None of these 17 denervated arteries showed the characteristic noradrenergic fluorescence seen at the medial-adventitial border of each control vessel.

Depletion of the intraneuronal noradrenaline stores:

In each of 13 experiments described in Chapter 5, a rabbit

was given reserpine (2.5 mg/Kg body weight) by intraperitoneal injection 24 hours prior to removal of the ear arteries for examination.

Venous occlusion plethysmography in man:

Blood flow in human limb segments was assessed by the technique of venous occlusion plethysmography which relates blood flow to changes in the volume of the limb segments under consideration. The theory and technique of the plethysmographic method have been critically appraised and found to be a valid measure of blood flow under normal haemodynamic conditions (Landowne and Katz, 1942; Formel and Doyle, 1957; Greenfield, 1960). For the purposes of the present studies, blood flow measurements were confined to the hand and the methods used in these investigations and the calculations employed were basically the same as those outlined by Barcroft and Swan (1953). The plethysmograph used was similar to that described by Greenfield (1954) and is shown in Appendix Fig. 4. It consisted of two separate chambers, an inner one into which the hand, enclosed in a thin rubber glove, was sealed and an outer one which acted solely as a water bath in order to maintain the constant desired temperature of 32^{o}C. Temperature adjustment was affected by adding water of appropriate temperature to the outer chamber and a mechanical stirrer prevented temperature layering effects.

Inflation and deflation of the pneumatic collecting cuffs

was achieved automatically by means of a sequence timer (Appendix Fig. 5) which electrically operated solenoid valves and so released air from a compressed air cylinder, using constant pressure valves. Care was taken to position the hands and forearms above heart level so that adequate venous drainage occurred during each phase of venous emptying. This ensured that the capacity vessels were relatively empty prior to each subsequent flow estimation.

The subjects for these experiments were normal volunteer medical students, aged from 20 to 29 years. The experiments were performed at laboratory temperatures ranging from 24°C to 28°C; the subjects lying recumbent on a couch for at least 30 minutes before the observations were made, during which time the recording apparatus was applied and the infusion needle inserted. Hand blood flow was measured as described above by obtaining three or four records of flow each minute. Intra-arterial drug infusions of 5 minutes duration were given into the brachial artery at the elbow of one side, through a 22-gauge needle connected by a length of polythene tubing to a mechanically driven syringe delivering 2 ml of solution per minute. Saline (0.9% w/v) was infused during the control periods and was also used as a vehicle for the drugs. The doses of the drugs were such that they did not produce systemic effects, making it possible to use the opposite uninfused limb as a control. Percentage changes in hand blood flow produced by the

sympathomimetic agents were determined from the averaged flow values during the 2 minutes before the drug infusion and the last 2 minutes of the infusion period, by which time the responses to the drugs had become stable. Allowance was made for spontaneous variation in the flow unrelated to drug action, by assuming that in the absence of each drug infusion, the infused and the control sides would have maintained the same relationship to each other as in the pre-infusion period (Duff, 1952).

C H A P T E R 3

THE HISTOCHEMICAL LOCALIZATION OF MONOAMINE OXIDASE

INTRODUCTION:

Extensive use has been made of the rabbit ear artery in studies on the control of vascular sensitivity, both in this thesis and in other investigations (de la Lande, Cannell and Waterson, 1966; de la Lande, Frewin and Waterson, 1967; de la Lande and Waterson, 1968a). One of the main purposes of this thesis has been to further explore the role of monoamine oxidase in vascular sensitivity. Accordingly, it was first considered necessary to investigate the distribution of the enzyme in the ear artery and this aspect was studied in some detail. More precise information about the location of monoamine oxidase in vascular tissue was also likely to have considerable implications concerning the actions of certain biogenic amines such as noradrenaline and tyramine on vascular sensitivity.

Earlier evidence concerning the presence of monoamine oxidase in the rabbit ear artery has been confined to reports by Thompson and Tickner (1951), Armin, Grant, Thompson and Tickner (1953), and Koelle and Valk, Jr (1954). Thompson and Tickner (1951) identified and assayed the enzyme by manometric and titrimetric methods in various isolated animal arteries (including the rabbit

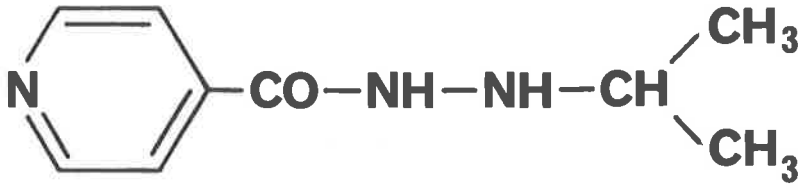
ear artery) from which the adventitia had been removed. In conjunction with Armin and Grant (Armin, Grant, Thompson and Tickner, 1953), they showed by the same technique that monoamine oxidase activity in the central artery of the rabbit ear was not reduced following sympathetic denervation of the ear. Koelle and Valk, Jr (1954), employing a hydrazine method, demonstrated the presence of monoamine oxidase in various tissues of the cat and some rabbit tissues. They found, *inter alia*, that the enzyme was present in the media of blood vessels, including the rabbit ear artery. This latter histochemical technique is, however, cumbersome and has largely been superseded by the tetrazolium method of Glenner, Burtner and Brown, Jr (1957).

Indirect evidence for the presence of monoamine oxidase in noradrenergic nerves and nerve terminals has been provided in recent years (Kopin and Gordon, 1962a and b, 1963; Kopin, 1964; Snyder, Fischer and Axelrod, 1965; Hamberger, Malmfors, Norberg and Sachs, 1964; Malmfors, 1965). It is emphasized that except in large arteries, the sympathetic nerve terminals are not present in the media of blood vessels, but tend to be concentrated in the adventitia at the medial-adventitial junction. This distribution of the noradrenergic motor innervation of vascular smooth muscle has been noted in various animals by means of the noradrenaline fluorescence technique (Falck, 1962; Norberg and Hamberger, 1964;

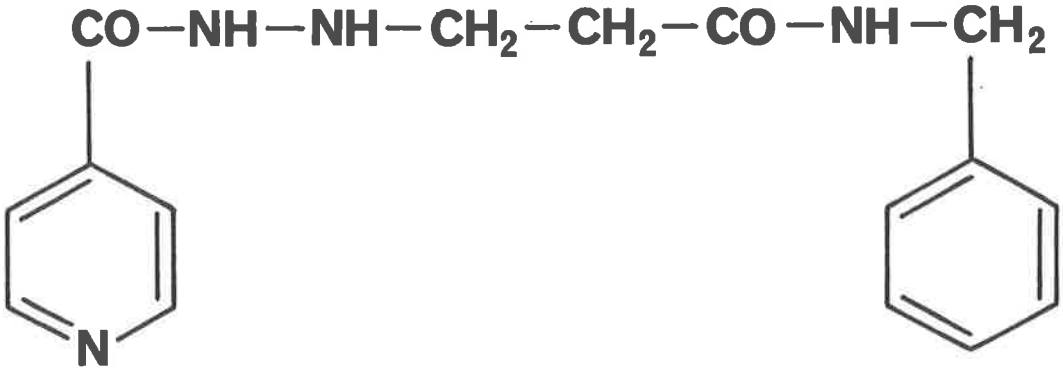
Fuxe and Sedvall, 1964, 1965; Waterson and Smale, 1967; de la Lande and Waterson, 1967). These findings agree with electron-microscopical studies of the innervation of blood vessels (Pease and Molinari, 1960; Lever and Esterhuizen, 1961; Rhodin, 1962; Lever, Graham, Irvine and Chick, 1965), demonstrating that the autonomic ground plexus is generally confined to the medial-adventitial border except in the largest muscular arteries (Keatinge, 1966).

Hence, in the light of the above information, it was of considerable importance to determine histochemically whether or not monoamine oxidase was similarly concentrated at the medial-adventitial border of the rabbit ear artery. So that physiological function could be related to histochemical monoamine oxidase activity in the artery wall, the effects of the monoamine oxidase inhibitors, iproniazid and nialamide (Fig. 3-1) were also recorded in this study. In addition, the results of chronic sympathetic denervation on the histochemical distribution of the enzyme in the wall of the rabbit ear artery were also examined.

In view of the evidence that bretylium is a weak reversible inhibitor of monoamine oxidase (Furchgott, 1964; Furchgott and Sanchez Garcia, 1966) and is accumulated by sympathetic nerves and nerve terminals (Boura and Green, 1959; Furchgott and Sanchez Garcia, 1966), it seemed possible that the action of bretylium in



IPRONIAZID



NIALAMIDE

Fig. 3-1 Formulae of iproniazid and nialamide.

protecting the monoamine oxidase in nerve terminals from irreversible inhibition by a drug such as iproniazid (Furchgott and Sanchez Garcia, 1966), might thus permit histological discrimination between intraneuronal and extraneuronal monoamine oxidase. Accordingly, this aspect of bretylium's action was investigated. Nialamide, however, was employed instead of iproniazid in the experiments described in this chapter.

MATERIALS AND METHODS:

The rabbit tissues examined were the central ear artery, the whole ear containing the vessels and nerves *in situ* and the kidney. The distribution of monoamine oxidase was demonstrated by the method of Glenner, Burtner and Brown, Jr (1957), with minor modifications. The original technique and the modifications employed have been described in detail in Chapter 2.

Monoamine oxidase inhibition:

(a) *In vivo:* Iproniazid (200 mg/Kg body weight) was administered intraperitoneally to the animal 24 hours prior to removal of the ear arteries.

(b) *In vitro:* Two arteries, one from each ear of the same rabbit, were excised and perfused by the double-cannulation technique which has been described in detail in Chapter 2. One artery was perfused for 1 hour with the monoamine oxidase inhibitor (either iproniazid 100 µg/ml or nialamide 100 µg/ml) by adding the

inhibitor to the Krebs bicarbonate perfusion solution and also to the extraluminal Krebs bicarbonate bathing solution. The inhibitor solution was replaced by drug-free solution after 1 hour and the artery was perfused for another 10 minutes to 2 hours before examining it for the presence of monoamine oxidase. The opposite ear artery was used as a control, in that it was not perfused with the inhibitor, but was perfused with drug-free Krebs bicarbonate solution for the same period of time.

Sympathetic denervation:

One central ear artery in each of 14 rabbits was denervated by prior removal of the respective superior cervical ganglion 9 to 24 days previously (12 rabbits), 30 days previously (1 rabbit) and 60 days previously (1 rabbit). The sterile surgical technique employed was that of de la Lande and Rand (1965). This method and the tests of the effectiveness of the denervation procedure have been described in detail in Chapter 2.

Bretylium effect:

Segments of ear arteries taken from the same rabbit respectively in each of 3 experiments, were incubated in media consisting of bretylium in Krebs bicarbonate solution (100 µg/ml, 500 µg/ml and 1 mg/ml) for 1 hour. Nialamide was then added to each solution so that the final nialamide concentration was 100 µg/ml and the bretylium concentration remained unchanged. Following

incubation for 1 hour, each solution was removed and replaced by bretylium alone (100 µg/ml, 500 µg/ml and 1 mg/ml, respectively) for 15 minutes. Bretylium was then washed out and replaced by Krebs bicarbonate solution for 15 minutes. The artery segments were then examined histochemically for their monoamine oxidase content. Two control artery segments were employed in each experiment. The first was treated with nialamide and Krebs bicarbonate solution in the absence of bretylium and the second was incubated in Krebs bicarbonate solution alone for the same period of time. All solutions were maintained at 37°C and were aerated with a gas mixture of 95% oxygen and 5% carbon dioxide.

RESULTS:

Rabbit ear artery:

Sections of the central ear artery, both *in situ* and after perfusion for several hours *in vitro* with Krebs bicarbonate solution, displayed an identical distribution of monoamine oxidase activity. Diffuse and some granular staining reaction was distributed uniformly throughout the media and was largely confined to this region of the artery wall. In particular, there was no evidence of more intense activity at the medial-adventitial border where the sympathetic nerve terminals are known to be concentrated. There was slight activity in the intima and also a slight patchy distribution of granular staining reaction in the adventitia, which was more

evident in the non-perfused arteries.

An example of an ear section with the central artery *in situ* is shown in Fig. 3-2. The appearance of this artery is typical of more than 40 such vessels which were examined and it shows the features described previously, with the addition of monoamine oxidase activity in large adjacent mixed somatic and autonomic nerve bundles, constituting part of the ventral auricular nerve of the ear. Unmyelinated postganglionic sympathetic axons were indistinguishable within these nerve bundles from myelinated somatic nerve fibres which showed considerable activity and it was not possible to determine whether or not the unmyelinated fibres were stained.

Figure 3-3 typifies the appearance of the 14 arteries which were denervated by removal of the ipsilateral superior cervical ganglion 9 to 60 days previously. The ear section shown previously in Fig. 3-2 was removed from the opposite ear of the same rabbit from which the denervated ear section shown in Fig. 3-3 was taken and thus acted as a control. Both the intensity and distribution of the staining in the artery walls of the 14 denervated arteries were indistinguishable from those of the corresponding (contralateral) control arteries. It will also be noted in Fig. 3-3 that monoamine oxidase staining in the mixed nerve bundles adjacent to the artery in each case was similarly unaltered by sympathetic denervation.

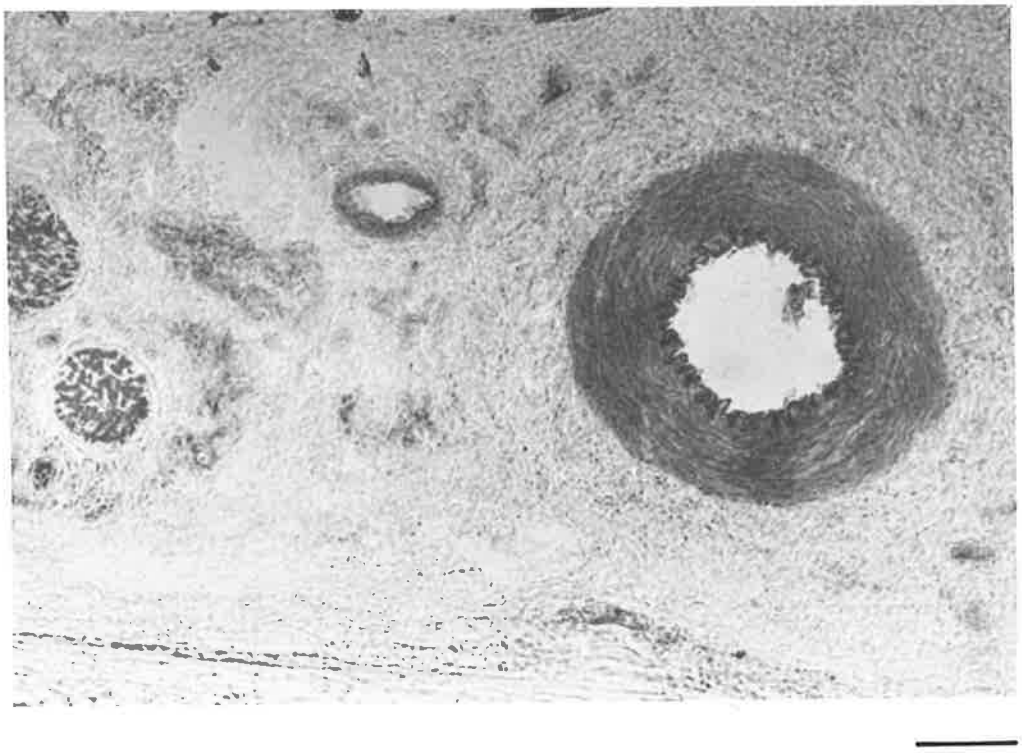


Fig. 3-2 Transverse section of the right ear with the central artery *in situ*, showing moderate diffuse staining reaction in the media of the vessel and staining also in large adjacent mixed nerve bundles of the ventral auricular nerve of the ear. This section is the control for the denervated ear section shown in Fig. 3-3.

Section thickness: 20 microns

Nitro-blue tetrazolium

Incubation time: 45 minutes

Scale: 100 microns

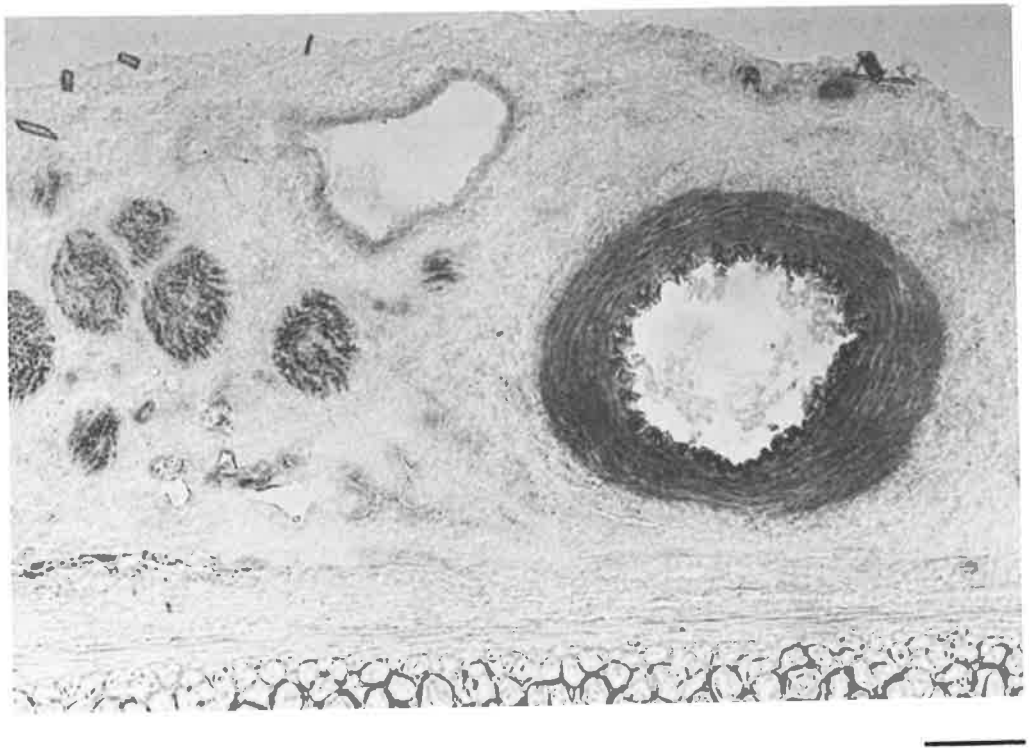


Fig. 3-3 Transverse section of the left ear with the central artery *in situ*, following sympathetic denervation 24 days previously. There is moderate staining reaction in the media of the vessel and staining also in large adjacent mixed nerve bundles of the ventral auricular nerve of the ear. The appearances are comparable with those of the control right ear section shown in Fig. 3-2.

Section thickness: 20 microns

Nitro-blue tetrazolium

Incubation time: 45 minutes

Scale: 100 microns

Figure 3-4 is an example of a control (untreated) artery which had been perfused for 1 hour with Krebs bicarbonate solution and it is typical of more than 60 control arteries which were examined. It was noted, however, that in sections of perfused and non-perfused arteries which had been incubated for the same time, the intensity of medial staining was less intense in the perfused vessels, indicating that perhaps a soluble component of the monoamine oxidase had been removed by the perfusion.

That the staining seen in the media was due to monoamine oxidase was indicated by the following tests:

1. In control arteries, perfused with Krebs bicarbonate solution, sections which were incubated without the tryptamine hydrochloride substrate showed a complete absence of staining reaction (Fig. 3-5).

2. The intensity of staining was markedly reduced in 10 arteries removed from 6 rabbits which had been pretreated with iproniazid (Fig. 3-6).

3. Similarly, staining was markedly reduced in more than 30 arteries which had been perfused for 1 hour with iproniazid (100 µg/ml) or with nialamide (100 µg/ml). An example of an artery which had been perfused with nialamide (100 µg/ml) is shown in Fig. 3-7. The artery shown in Fig. 3-4 was the control vessel for this nialamide-treated artery. A markedly reduced intensity of monoamine

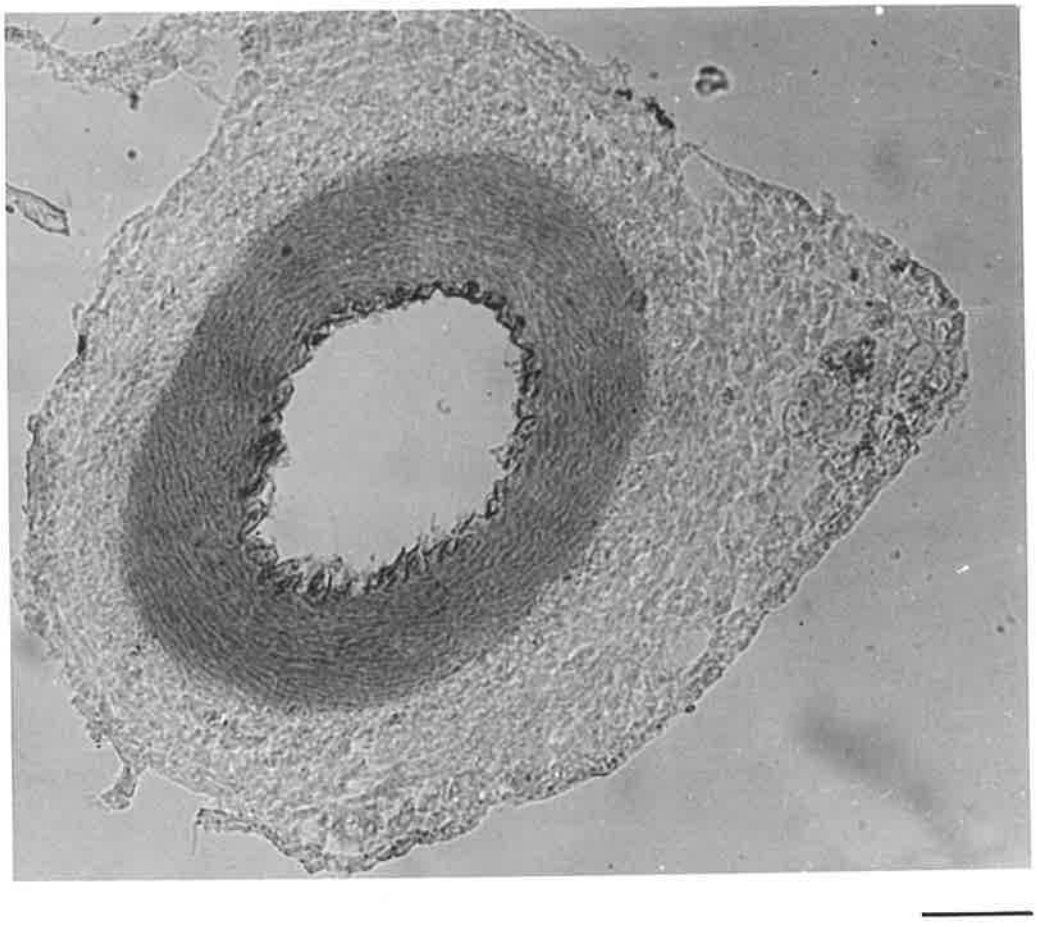


Fig. 3-4 Transverse section of a control ear artery which had been perfused for 1 hour with Krebs bicarbonate solution, showing diffuse staining reaction in the media.

Section thickness: 20 microns

Nitro-blue tetrazolium

Incubation time: 2 hours

Scale: 100 microns

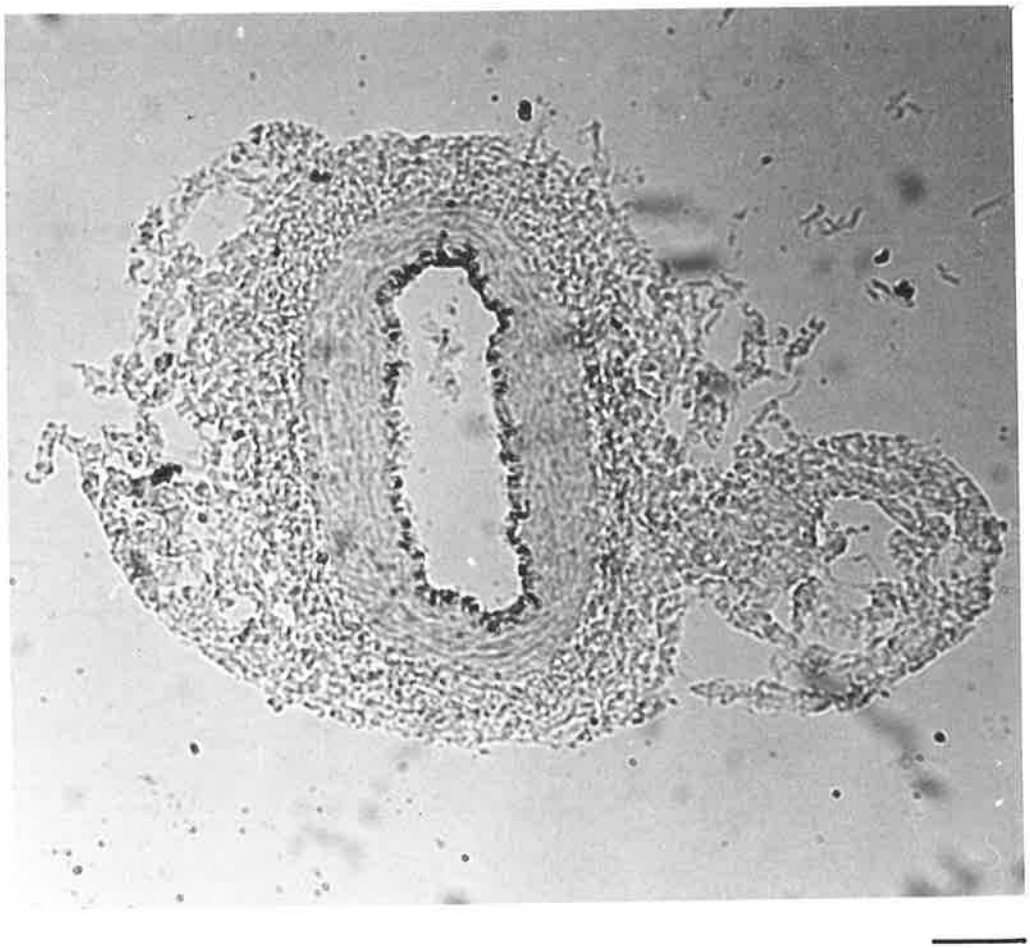


Fig. 3-5 Transverse section of a control ear artery, perfused with Krebs bicarbonate solution for 1 hour and subsequently incubated in the absence of the tryptamine hydrochloride substrate, showing absence of staining reaction in the media.

Section thickness: 20 microns

Nitro-blue tetrazolium

Incubation time: 2 hours

Scale: 100 microns

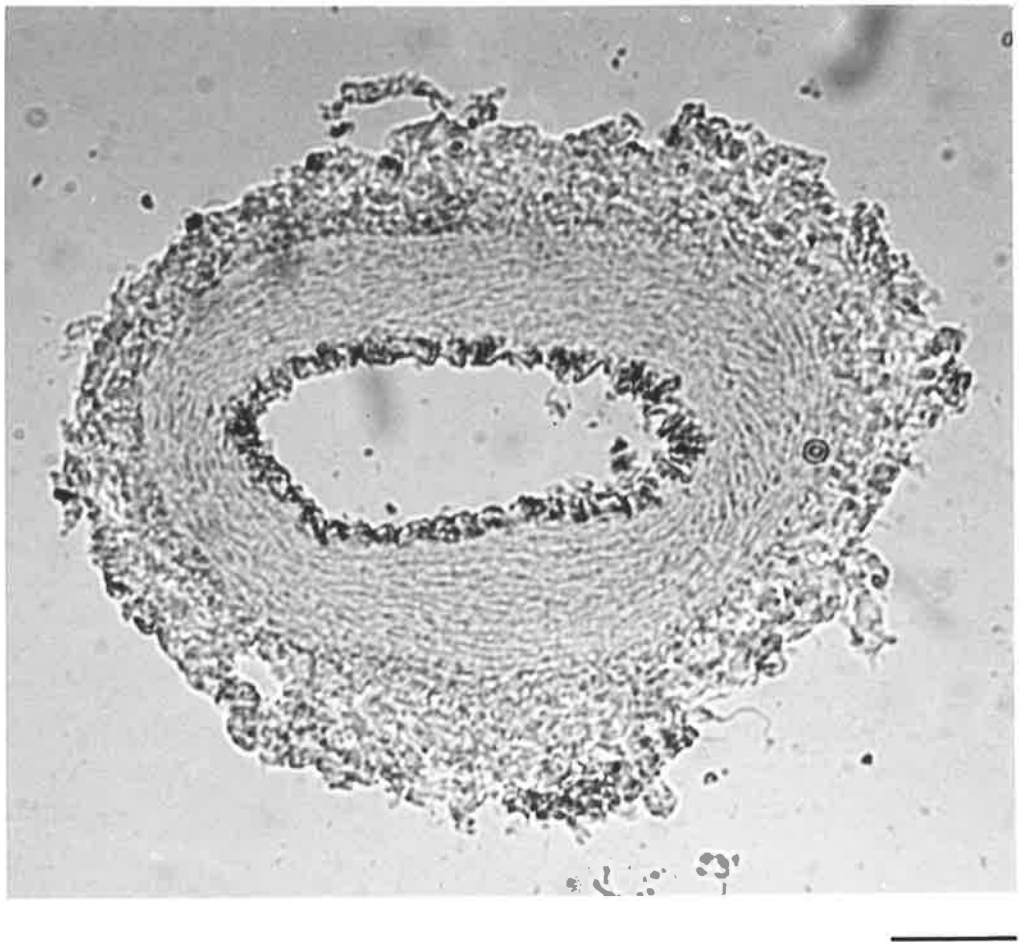


Fig. 3-6 Transverse section of a Krebs-perfused ear artery removed from a rabbit which had been pretreated intraperitoneally with iproniazid (200 mg/Kg body weight) 24 hours previously, showing markedly reduced staining reaction in the media.

Section thickness: 20 microns

Nitro-blue tetrazolium

Incubation time: 2 hours

Scale: 100 microns

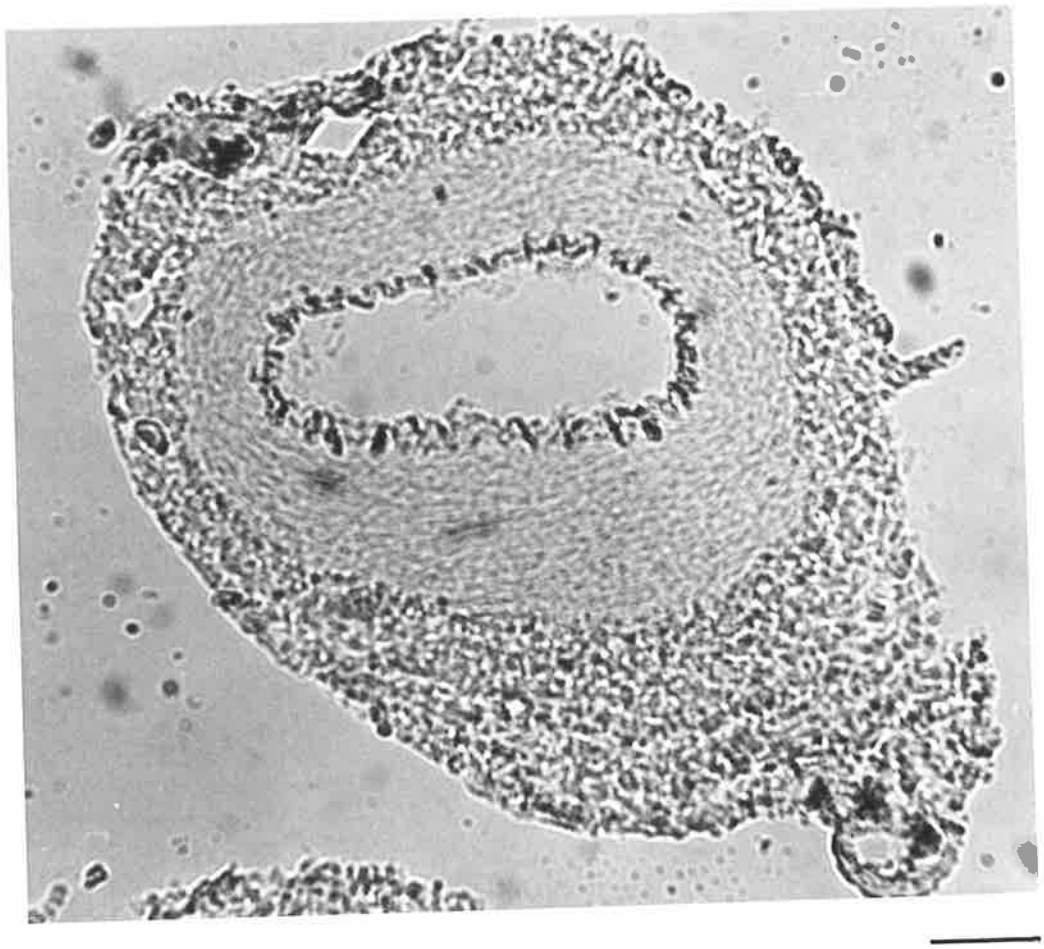


Fig. 3-7 Transverse section of an ear artery which had been perfused with nialamide (100 µg/ml) for 1 hour, showing markedly reduced staining reaction in the media.

Section thickness: 20 microns

Nitro-blue tetrazolium

Incubation time: 2 hours

Scale: 100 microns

oxidase activity in the media compared with that in the control artery in each case, was also seen in arteries perfused with iproniazid in concentrations down to 40 µg/ml and nialamide in a concentration of 10 µg/ml produced a moderate reduction in the intensity of staining.

4. Incubation of Krebs-perfused control arteries in solutions containing tetranitro-blue tetrazolium in place of nitro-blue tetrazolium signified an identical distribution of monoamine oxidase activity in the media (Fig. 3-8). The formazan precipitate produced in this case, however, was brown in colour.

Bretylium effect:

Attempts to define the presence of monoamine oxidase within the sympathetic nerve terminals of the ear artery were unsuccessful in that the appearance of sections of arteries treated with both bretylium and nialamide were indistinguishable from those of arteries treated with nialamide alone. This indicated that in this experimental situation, bretylium had failed to protect either the intraneuronal or extraneuronal enzyme from inhibition by nialamide.

Kidney:

Histochemical sections of kidney tissue (Fig. 3-9) displayed intense blue-staining monoamine oxidase activity, both diffuse and granular, in the epithelium of the distal tubules, with

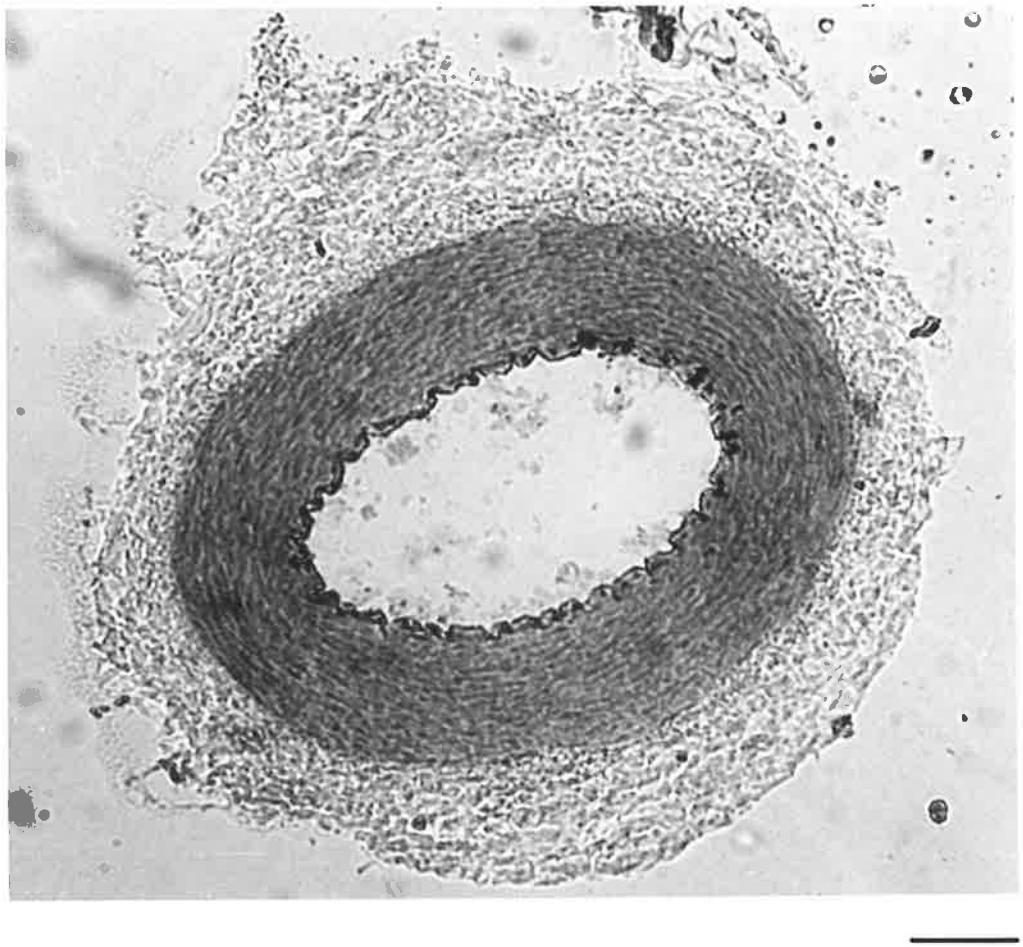


Fig. 3-8 Transverse section of a control ear artery which had been perfused for 1 hour with Krebs bicarbonate solution, showing diffuse staining reaction in the media.

Section thickness: 20 microns

Tetranitro-blue tetrazolium

Incubation time: 2 hours

Scale: 100 microns

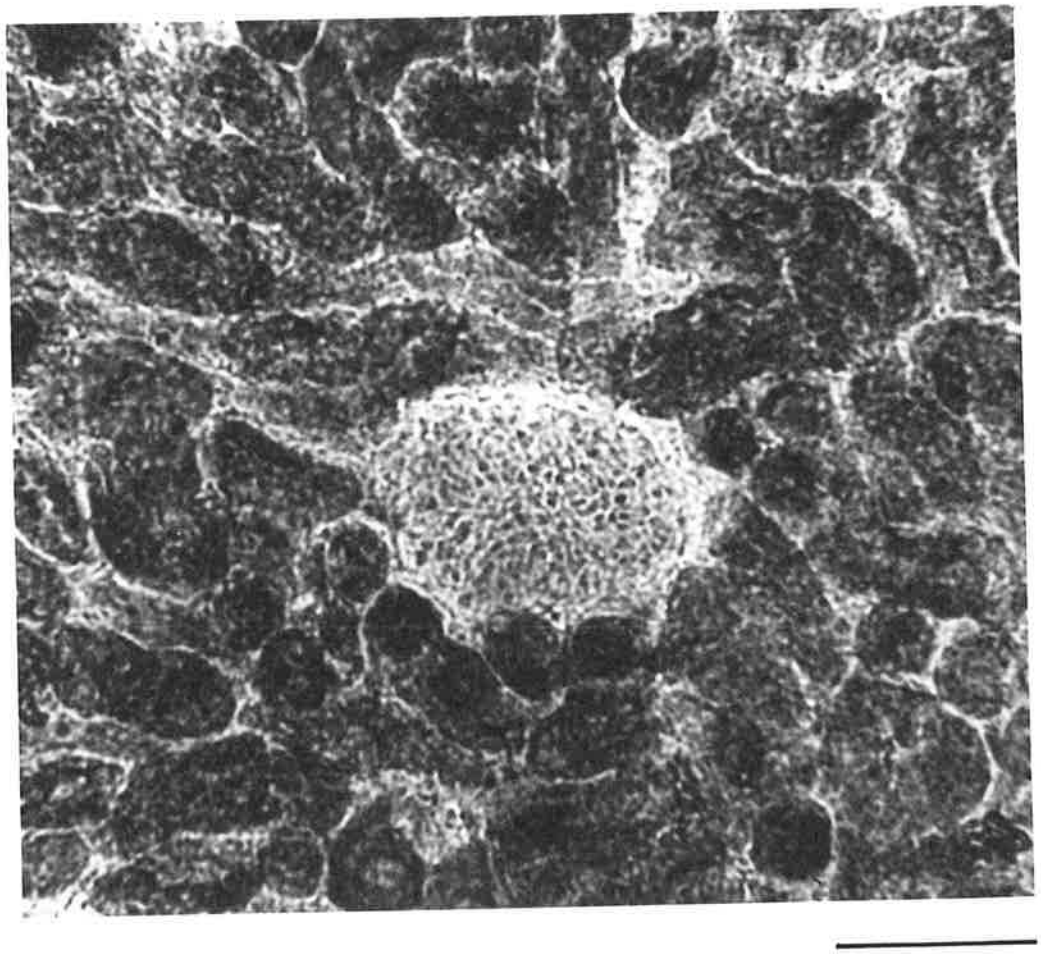


Fig. 3-9 Section of renal cortex showing intense diffuse and granular staining of the distal tubules, with moderate staining reaction in the proximal tubules. Only faint glomerular epithelial reaction is visible.

Section thickness: 20 microns

Nitro-blue tetrazolium

Incubation time: 45 minutes

Scale: 100 microns

moderate staining reaction in the proximal tubules and cortical and outer medullary portions of the collecting ducts. Only faint glomerular activity was seen and the contents of the more central medullary regions were unstained. Sections of renal cortex, incubated in the absence of the tryptamine hydrochloride substrate, showed absence of monoamine oxidase activity (Fig. 3-10) and sections of renal cortex taken from animals which had been pretreated with iproniazid, showed marked inhibition of monoamine oxidase staining reaction (Fig. 3-11). These findings tended to confirm that the staining reaction seen in the renal cortex was, in fact, due to the presence of monoamine oxidase.

DISCUSSION:

The observation of Koelle and Valk, Jr (1954) that monoamine oxidase, identified by their own histochemical procedure, is present throughout the media of the central artery of the rabbit ear, has been confirmed by the current findings which were obtained by the technique of Glenner, Burtner and Brown, Jr (1957). Monoamine oxidase present in the media, is extraneuronal, as distinct from the intraneuronal enzyme situated inside the axons of the postganglionic sympathetic nerves.

Although there was slight granular monoamine oxidase activity in the adventitia of the rabbit ear artery, significant amounts of the enzyme were not seen at the medial-adventitial border.

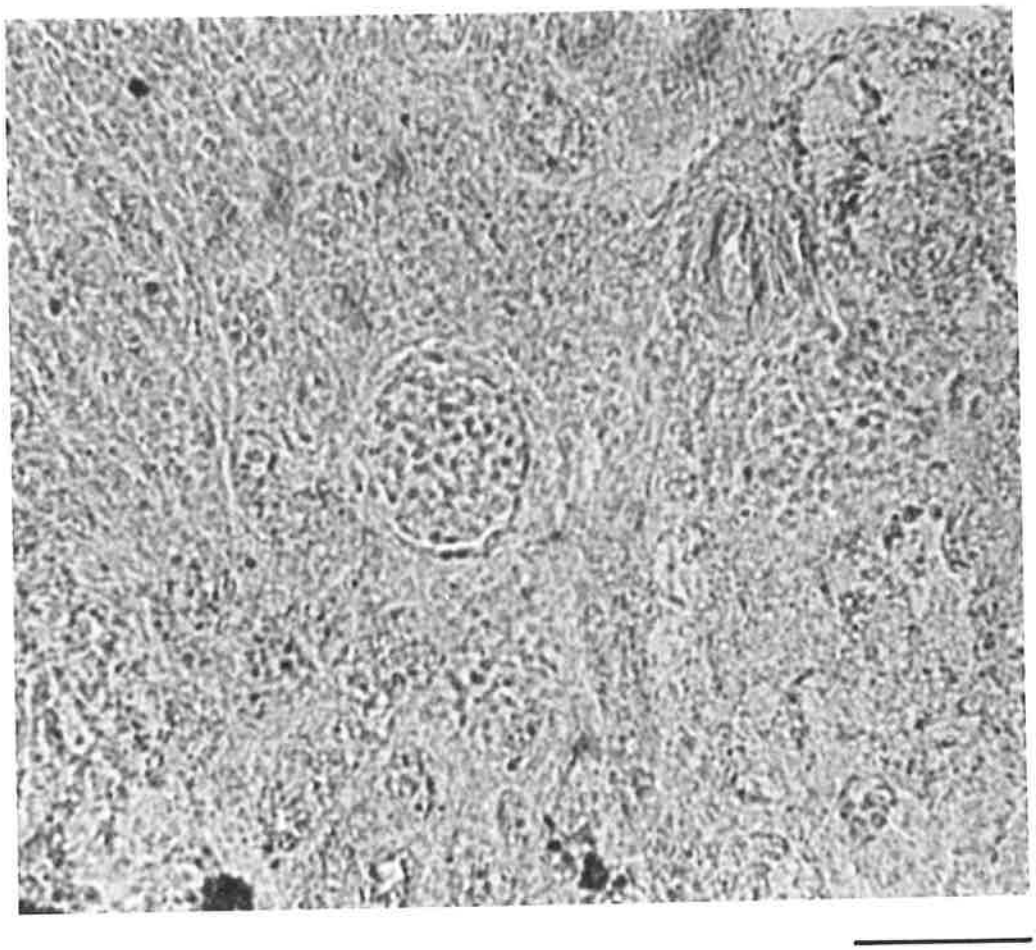


Fig. 3-10 Section of renal cortex incubated in the absence of the tryptamine hydrochloride substrate, showing complete absence of staining reaction.

Section thickness: 20 microns

Nitro-blue tetrazolium

Incubation time: 45 minutes

Scale: 100 microns

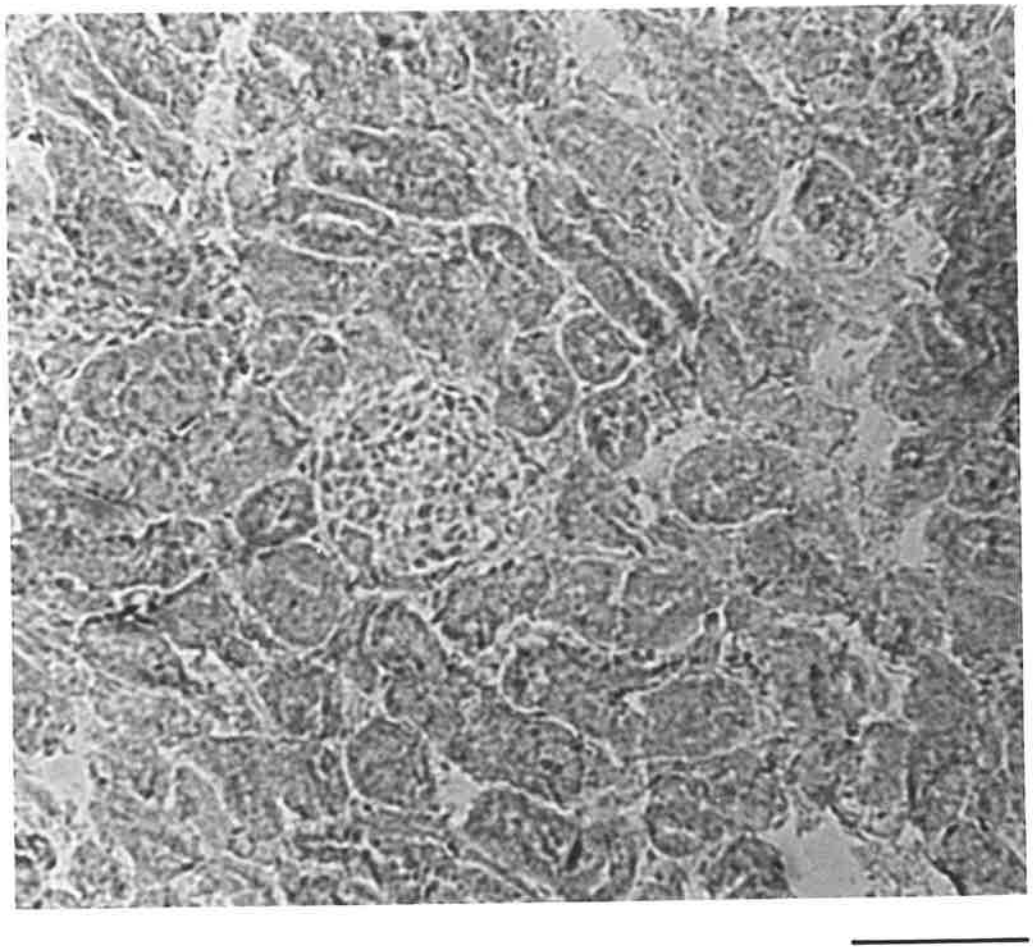


Fig. 3-11 Section of renal cortex following intraperitoneal pretreatment of the rabbit 24 hours previously with iproniazid (200 mg/Kg body weight), showing marked inhibition of monoamine oxidase activity.

Section thickness: 20 microns

Nitro-blue tetrazolium

Incubation time: 45 minutes

Scale: 100 microns

This is surprising in view of the evidence provided in the Introduction to this chapter, that the sympathetic nerve terminals containing monoamine oxidase are concentrated at this site in arteries. In addition, small mixed somatic and autonomic nerve bundles lie in the tissue of the ear in the vicinity of the central artery and these nerve bundles also showed fairly intense monoamine oxidase activity. It was not possible to distinguish between myelinated fibres and unmyelinated postganglionic sympathetic nerve axons within these nerve bundles.

There have been conflicting reports concerning the histochemical staining of monoamine oxidase in myelinated and unmyelinated nerve fibres in different animal species when they were examined by the histochemical technique of Glenner, Burtner and Brown, Jr (1957). These workers observed in the guinea-pig that unmyelinated autonomic fibres but not myelinated fibres exhibited the characteristic staining. However, myelinated nerve fibres were noted to be stained in rat ganglia (Härkönen, 1964) and in rat sciatic nerves (Dahlström, Jonason and Norberg, 1969). Shantha-veerappa and Bourne (1964) indicated that strong monoamine oxidase activity was present in the myelin sheath of rabbit optic and oculomotor nerves but was absent in the axons. Koelle and Valk, Jr (1954), employing their own histochemical technique, referred to the significant species differences in the relative concentrations

of monoamine oxidase in different comparable tissues.

A possible explanation for the virtual absence of intraneuronal monoamine oxidase staining reaction in the sympathetic nerve terminals situated at the medial-adventitial border of the artery wall of the rabbit ear, may be that the sympathetic axons in this situation are very thin and the sensitivity of the present method may not allow the detection of the enzyme by the resolution of the light microscope. This was the explanation given by Almgren, Andén, Jonason, Norberg and Olson (1966) for their inability to detect histochemical monoamine oxidase activity in the sympathetic nerve terminals in rat salivary glands when examined by the method of Glenner, Burtner and Brown, Jr (1957). Alternatively, the affinity of intraneuronal monoamine oxidase for the tryptamine hydrochloride substrate employed may be less than that of extraneuronal monoamine oxidase, so that the enzyme within these thin postganglionic sympathetic axons is not revealed to any significant extent.

It is of interest that Fujiwara, Tanaka, Hikosaka and Okegawa (1966) have drawn attention to the lack of correlation between the cytological distribution of monoamine oxidase activity and noradrenaline fluorescence in the salivary glands of the dog, indicating that monoamine oxidase activity does not necessarily represent the presence of noradrenergic nerve fibres. Lukáš and

Čech (1966) have also observed in ocular tissues of the pig, dog and rat, that not all noradrenergic nerve fibres, as indicated by noradrenaline fluorescence, have been revealed by histochemical examination of their monoamine oxidase content. This finding applied also to the plexuses of sympathetic nerve terminals at the medial-adventitial border of blood vessels in various tissues of the eyes of these animals.

There have been varying reports concerning the effect of sympathetic denervation on monoamine oxidase activity in different tissues. Snyder, Fischer and Axelrod (1965) observed a slight biochemical reduction of monoamine oxidase in the rat pineal gland and submaxillary gland; and Almgren, Andén, Jonason, Norberg and Olson (1966) have also reported a biochemical reduction in rat salivary glands. On the other hand, Strömblad (1956) estimated the monoamine oxidase activity in salivary glands of the cat and found no reduction in the enzyme activity after sympathetic denervation. In addition, the results of Fujiwara, Tanaka, Hikosaka and Okegawa (1966), employing salivary glands of the dog, indicated by the histochemical method of Glenner, Burtner and Brown, Jr (1957), that removal of the superior cervical ganglion did not significantly affect the monoamine oxidase activity. They also measured the enzyme content manometrically and their findings were similar to the above when tyramine was used as the substrate. However, when

serotonin was employed as the substrate, monoamine oxidase activity was significantly reduced.

In the present study, employing the histochemical method of Glenner, Burtner and Brown, Jr (1957), no diminution of extra-neuronal monoamine oxidase activity in the media of the central artery of the rabbit ear could be detected after sympathectomy. This finding is in agreement with the earlier biochemical results of Armin, Grant, Thompson and Tickner (1953) since these workers were also unable to demonstrate any significant change in monoamine oxidase content of the rabbit ear artery following sympathetic denervation. Most of the enzyme content of the artery wall was extraneuronal and was situated in the smooth muscle layer. There was also no significant diminution of the staining reaction in the mixed nerve bundles of the rabbit ear following removal of the ipsilateral superior cervical ganglion. However, because the unmyelinated postganglionic noradrenergic axons were not discernible amongst the myelinated nerve fibres in the nerve bundles of the ear, it was not possible to form any conclusion concerning the effect of sympathectomy on intraneuronal monoamine oxidase in this situation. It has also been observed, by the histochemical method of Glenner, Burtner and Brown, Jr (1957), that lumbar sympathectomy in the rat did not significantly reduce the monoamine oxidase content of unligated sciatic nerves (Dahlström, Jonason and Norberg, 1969). An

explanation which may account for these latter findings concerning the nerves of the rabbit ear and the sciatic nerves of the rat is that the monoamine oxidase content of the postganglionic (unmyelinated) sympathetic axons constitutes such a small proportion of the total enzyme content of these mixed motor, sensory and autonomic nerves, that any diminution of monoamine oxidase content in the autonomic nerves following sympathetic denervation would not be visible.

SUMMARY

1. The histochemical localization of monoamine oxidase has been studied in detail in the rabbit ear artery. This artery has been used extensively in studies on the control of vascular sensitivity and the value of precise information regarding the distribution of monoamine oxidase in this vessel is in the interpretation of the various theories of vascular sensitivity to noradrenaline and tyramine which have been proposed.

2. Extraneuronal monoamine oxidase was demonstrated mainly throughout the media of the artery and its concentration at this site was not perceptibly altered by sympathetic denervation.

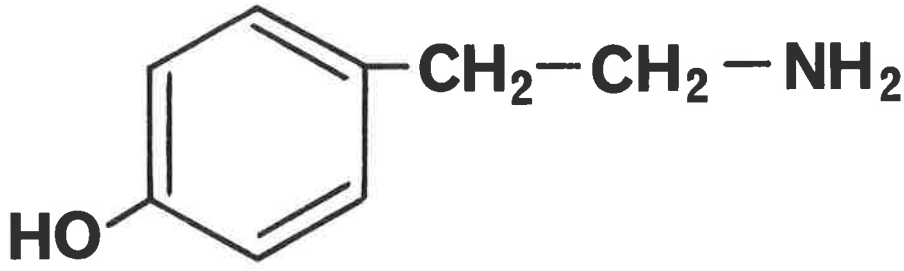
3. The failure to demonstrate any accumulation of the enzyme at the medial-adventitial border is surprising in view of the fact that the sympathetic nerve terminals, which contain intraneuronal monoamine oxidase, are concentrated at this site. However, the sympathetic nerve terminals are very small and the sensitivity of the present method may be insufficient to enable them to be detected by the resolution of the light microscope. Alternatively, the affinity of intraneuronal monoamine oxidase for the tryptamine hydrochloride substrate may be less than that of extraneuronal monoamine oxidase.

C H A P T E R 4

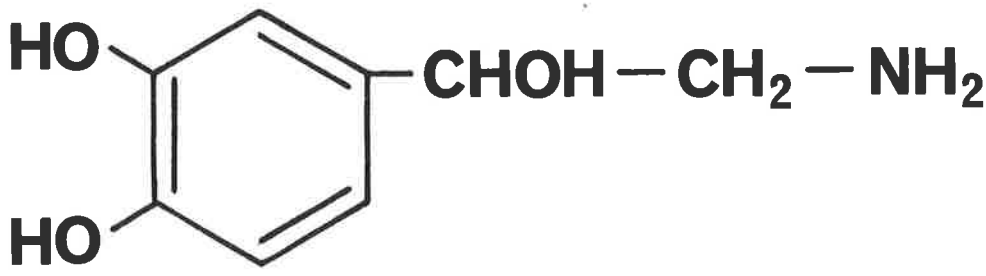
THE EFFECT OF MONOAMINE OXIDASE ON THE ACTION OF TYRAMINE

INTRODUCTION:

Considerable evidence has accrued (Burn and Rand, 1958; Axelrod, Gordon, Hertting, Kopin and Potter, 1962; Trendelenburg, Muskus, Fleming and Gomez Alonso de la Sierra, 1962a and b; Muscholl, 1966) indicating that the sympathomimetic amine tyramine (Fig. 4-1) has largely an indirect action on smooth muscle and that the indirect component is mediated by release of noradrenaline (Fig. 4-1) from the noradrenergic storage structures in the sympathetic nerve terminals. In agreement with this evidence was the detailed study of de la Lande and Waterson (1968a) which indicated that the response of the rabbit ear artery to extraluminally-applied tyramine was mainly indirect and mediated by the sympathetic nerve terminals at the medial-adventitial border of the artery. The indirect component was found to play a less prominent part in the responses to intraluminally-applied tyramine than in those to extraluminally-applied tyramine. These authors also observed that the artery was normally much more sensitive to extraluminal tyramine than to intraluminal tyramine and they suggested that this was due to the failure of intraluminal tyramine to penetrate as readily as



TYRAMINE



NORADRENALINE

Fig. 4-1 Formulae of tyramine and noradrenaline.

extraluminal tyramine to the region of the sympathetic nerve terminals. Three possible explanations for the lower sensitivity of the artery to intraluminal tyramine were considered, namely: (1) a permeability barrier located somewhere between the intima and the medial-adventitial border of the artery; (2) a major site of loss (possibly represented by monoamine oxidase in the media) located between the intima and the medial-adventitial border; and (3) dilution of the intraluminally-applied tyramine in the vicinity of the medial-adventitial border by the tyramine-free solution bathing the adventitia externally.

In the present study, the possible role of monoamine oxidase has been explored in some detail. As described in Chapter 3, monoamine oxidase has been shown by a modification of the specific histochemical technique of Glenner, Burtner and Brown, Jr (1957), to be largely confined to the media of the central ear artery of the rabbit and it was not seen histochemically to be concentrated in the region of the sympathetic nerve terminals which are known to be concentrated exclusively at the medial-adventitial border of this artery (Waterson and Smale, 1967; de la Lande and Waterson, 1967). However, convincing indirect evidence has accumulated (Kopin and Gordon, 1962a and b, 1963; Kopin, 1964; Snyder, Fischer and Axelrod, 1965; Hamberger, Malmfors, Norberg and Sachs, 1964; Malmfors, 1965), indicating

that the enzyme is in fact present within noradrenergic nerves and nerve terminals.

The localization of monoamine oxidase in the media and its probable extraneuronal distribution in this region imply that intraluminal tyramine, but not extraluminal tyramine, must diffuse through the region of the artery wall containing the enzyme, in order to reach the nerve terminals. Hence the morphological distribution of the sympathetic nerve terminals and monoamine oxidase in the artery wall, gives rise to the possibility that metabolism by monoamine oxidase plays a more important role in the response to intraluminal tyramine than in the response to extraluminal tyramine. In order to investigate this hypothesis, the effects of monoamine oxidase upon the actions of intraluminal and extraluminal tyramine have been examined and the relationship between these effects and the distribution of the enzyme in the artery wall has been explored.

MATERIALS AND METHODS:

Perfusion of the artery:

The method of isolating the central artery of the rabbit ear and the conditions of perfusing the vessel, using the double-cannulation technique, have been described in detail in Chapter 2. Double-cannulation of the artery enabled comparisons to be made between vasoconstrictor responses to tyramine added extraluminally to the extraluminal bathing fluid and the responses to tyramine

applied intraluminally by addition to the intraluminal perfusion solution (via the intraluminal reservoir).

Estimation of sensitivity:

Method 1. In earlier experiments, constrictor responses to repeated individual doses of intraluminal and extraluminal tyramine were recorded by their alternate application to the artery at intervals of 10 to 20 minutes following washout of the preceding dose. de la Lande and Waterson (1968a) commented on some of the problems encountered by using individual doses of tyramine. One of the major difficulties was that the sensitivity to extraluminal tyramine, and less frequently that to intraluminal tyramine, progressively declined throughout the period of perfusion of the artery. When the decline in sensitivity was gradual, for example, amounting to less than 20% of the height of consecutive tyramine responses, dose-response curves to intraluminal and extraluminal tyramine were obtained in the same manner as described for noradrenaline by de la Lande, Cannell and Waterson (1966), and de la Lande, Frewin and Waterson (1967), and as described in Chapter 2. The ratio of the equipotent intraluminal and extraluminal tyramine concentrations (sensitivity ratio) was determined at an arbitrarily-chosen response height of 60 mm of mercury. However, when the decline in sensitivity of the artery to tyramine was more rapid, the mean of the first few responses only was used in estimating the

respective sensitivity ratio. In later experiments, efforts were made to overcome the problems inherent with individual doses of tyramine and Method 2 describes procedures which were subsequently employed.

Method 2. Initially doses of tyramine were cumulated in order to reduce the time required to obtain dose-response curves. This procedure, however, was not entirely satisfactory in a number of arteries where the peak value of constriction was followed by a decline or fluctuation in the level of the response prior to administration of the next cumulative tyramine dose. Therefore, in subsequent experiments, cumulation was commenced at subthreshold doses so that the threshold dose could be determined. The respective threshold doses were used to estimate the ratio of the sensitivities of the artery to intraluminal and extraluminal tyramine.

In detail, the method employed was as follows: Tyramine was added to the extraluminal bathing solution in a subthreshold concentration of 0.01 µg/ml and the concentration was progressively increased at approximately 5 minute intervals by a factor of 2 or 2.5 times. An increase in perfusion pressure above the resting level (base-line) of magnitude 2 mm or greater was regarded as the threshold response. Usually the dose of tyramine was further increased to provide several responses of greater magnitude. Fifteen minutes after extraluminal tyramine washout

tyramine was added to the perfusion reservoir in a subthreshold dose and the threshold dose for intraluminal tyramine was determined. The threshold was estimated as the range between the dose which first elicited a response and the immediately preceding dose. In order to take into account a possible bias in the estimates of relative threshold doses due to changes in sensitivity to tyramine with time of perfusion, the order in which intraluminal and extraluminal tyramine were applied was reversed in one half of the experiments.

Monoamine oxidase inhibition:

The effects of monoamine oxidase inhibition on the artery and on the sensitivity of the artery to tyramine were examined in the following ways:

(a) *In vivo:* Iproniazid 200 mg/Kg was administered to the rabbit by intraperitoneal injection 24 hours prior to removal of the ear arteries. The constrictor potencies of tyramine in these inhibited arteries were compared with the potencies prevailing in control ear arteries removed from untreated rabbits.

(b) *In vitro:* Two arteries, one from each ear of the same rabbit in each case, were excised. One artery was perfused for 1 hour with the monoamine oxidase inhibitor, nialamide (100 µg/ml), by simultaneously adding it to the intraluminal perfusion solution and also to the extraluminal bathing solution. These

solutions were replaced by drug-free media after 1 hour and the artery was perfused with Krebs bicarbonate solution for a further 10 minutes prior to examination of its sensitivity to tyramine. The opposite ear artery was used as a control in that it was not perfused with the inhibitor but was perfused with drug-free Krebs bicarbonate solution for the same period of time (1 hour). Following the 10 minute washout period, sensitivities of both arteries to intraluminal and extraluminal tyramine were determined by Method 2.

(c) At the conclusion of most of these experiments, the arteries were examined histochemically for the presence of monoamine oxidase.

Histochemistry:

The distribution of monoamine oxidase was examined histochemically by the method of Glenner, Burtner and Brown, Jr (1957) as described in detail in Chapter 2.

RESULTS:

Iproniazid in vivo:

Individual responses to tyramine were examined in 10 arteries taken from 6 rabbits which had received the monoamine oxidase inhibitor iproniazid (200 mg/Kg body weight) by intraperitoneal injection 24 hours previously. The responses of these pretreated arteries were qualitatively similar to those of 14 control arteries removed

from untreated rabbits (Fig. 4-2) and have been described previously by de la Lande and Waterson (1968a). Thus, in most arteries, tyramine caused a rapid increase in perfusion pressure which was sustained at or near its maximum value for the period of contact of drug and artery (4 to 10 minutes). Occasionally the usual smooth response was interrupted by rapid fluctuations in the perfusion pressure (Fig. 4-3). Similar oscillations in the perfusion pressure were also seen in control (untreated) arteries, but were more commonly observed after monoamine oxidase inhibition.

These iproniazid-pretreated arteries also resembled control arteries in that they exhibited declining sensitivity to extraluminal tyramine and to a lesser extent to intraluminal tyramine throughout the course of the perfusion period of 3 to 4 hours. The responses of pretreated arteries did, however, differ from those of untreated arteries in one respect; namely, that the return of the perfusion pressure to its resting value following intraluminal and extraluminal tyramine washout was much slower in the pretreated arteries. This same phenomenon was also observed in the *in vitro* iproniazid and nialamide experiments which are described subsequently.

The main effect of iproniazid-pretreatment was to enhance the sensitivity of the artery irrespective of whether the tyramine was applied to the internal or external surface of the artery.

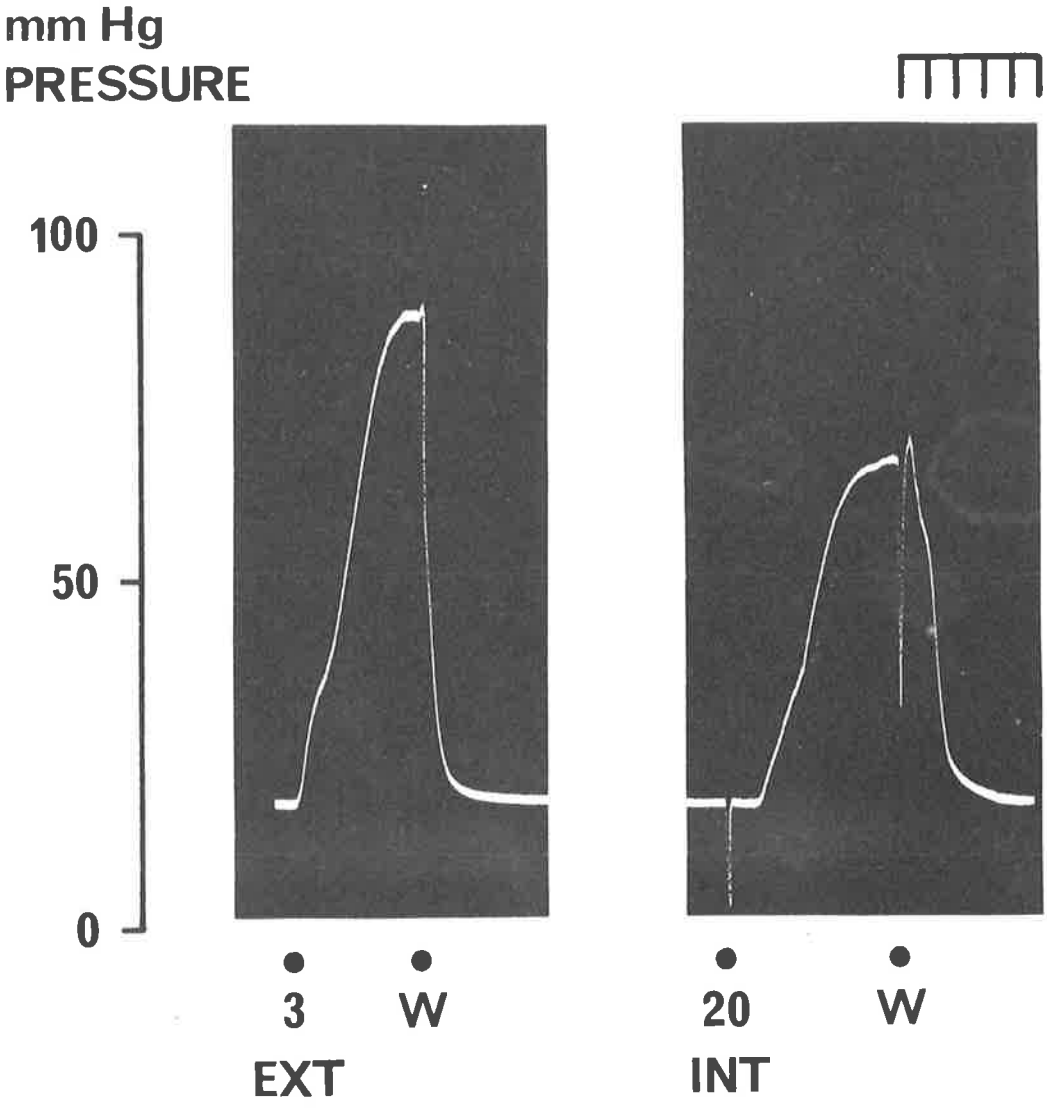


Fig. 4-2 Responses of an untreated control artery to extraluminal (EXT) and intraluminal (INT) doses of tyramine.

The numerals indicate dosage (µg/ml) of tyramine and W refers to tyramine washout.

Time trace: minutes.

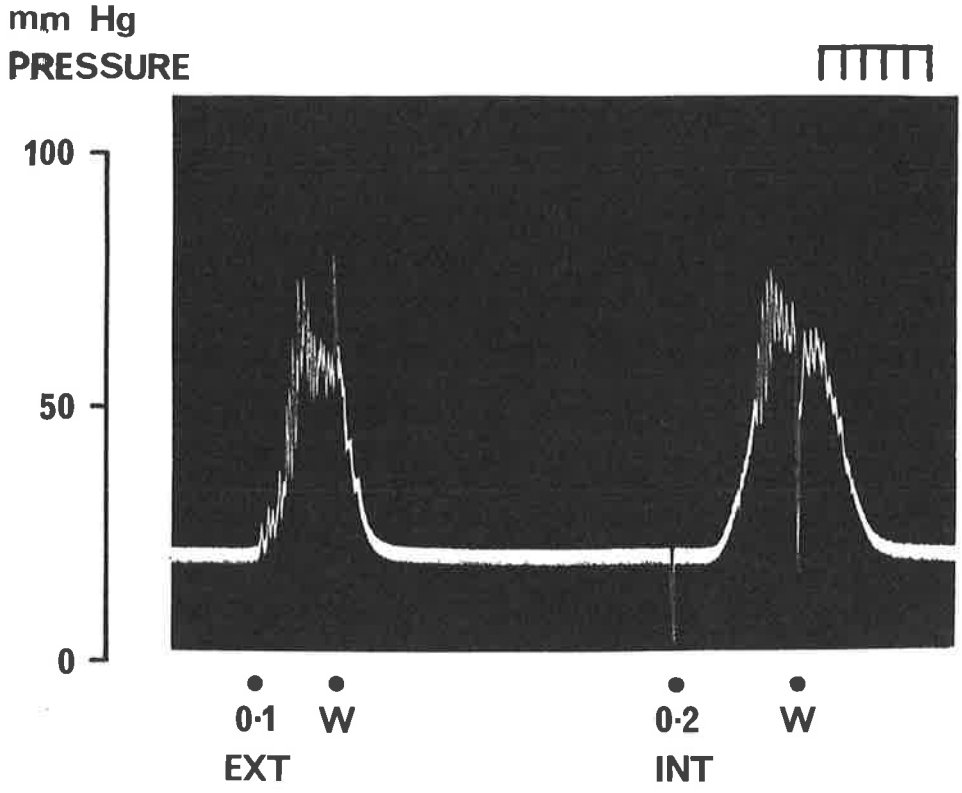


Fig. 4-3 Fluctuating constrictor responses to two consecutive extraluminal (EXT) and intraluminal (INT) doses of tyramine in an *in vivo* iproniazid-pretreated artery.

The numerals indicate dosage (µg/ml) of tyramine and W refers to tyramine washout.

Time trace: minutes.

This is illustrated by Table 4-1 which shows the individual intraluminal and extraluminal tyramine doses eliciting responses of magnitude 60 mm of mercury in 7 iproniazid-pretreated arteries and in 10 untreated (control) arteries. However, Table 4-2 which shows the mean values of these doses, and Table 4-3 which expresses the increases in sensitivity calculated from these mean values, both indicate that the average gain in sensitivity to intraluminal tyramine (60 fold) was considerably greater than that to extraluminal tyramine (12 fold).

Iproniazid in vitro:

Both ear arteries from each of 9 rabbits were removed and one artery in each case was perfused *in vitro* with iproniazid (100 µg/ml) for 1 hour. The opposite ear artery in each rabbit was simultaneously perfused with Krebs bicarbonate solution for the same time. The sensitivity of each artery to single doses of tyramine was then examined. This procedure, utilizing simultaneous perfusion of paired ear arteries, was adopted in an attempt to improve the precision of the comparisons between control and treated arteries, since it permitted the artery from the opposite ear to be used as a control. The effect of iproniazid *in vitro* was also to enhance the sensitivity of the artery to intraluminal and extraluminal tyramine, the gain in sensitivity being much greater with intraluminal tyramine. However, some of the comparisons were not

TABLE 4-1

Sensitivity of ear arteries to tyramine.

Doses of intraluminal and extraluminal tyramine (µg/ml) producing responses of 60 mm of mercury.

Experiment Number		1	2	3	4	5	6	7	8	9	10
Control (untreated animals)	Intraluminal tyramine	22	24	>100	5.0	3.7	24	6.2	36	40	19
	Extraluminal tyramine	1.6	6.4	2.0	0.76	0.77	1.6	1.7	1.6	3.1	4.0

Experiment Number		11	12	13	14	15	16	17
Iproniazid *in vivo* (200 mg/Kg IP)	Intraluminal tyramine	0.40	0.19	0.54	0.28	0.12	0.74	1.0
	Extraluminal tyramine	0.08	0.40	0.35	0.07	0.10	0.16	0.17

TABLE 4-2

Equipotent concentrations of tyramine (μg/ml)

Route of administration of tyramine	Tyramine doses producing responses of 60 mm of mercury	
	Control	Iproniazid *in vivo*
Intraluminal	28 (37) (19)	0.47 (0.59) (0.35)
Extraluminal	2.4 (2.9) (1.8)	0.19 (0.24) (0.14)

The values shown are the arithmetic means of the data quoted in Table 4-1. The figures in brackets refer respectively to mean + standard error and mean - standard error.

TABLE 4-3

Gain in sensitivity to intraluminal and extraluminal tyramine produced by iproniazid in vivo, expressed as the sensitivity ratio, $\frac{Control}{Iproniazid\ in\ vivo}$.

Route of administration of tyramine	Sensitivity ratio
Intraluminal	60
Extraluminal	12

These values of intraluminal and extraluminal sensitivity change were calculated from the arithmetic means quoted in Table 4-2.

entirely satisfactory, because the sensitivity to extraluminal and intraluminal tyramine declined even more rapidly after iproniazid treatment. In an attempt to reduce the time required to establish dose-response curves, the doses of tyramine were cumulated in later experiments (Fig. 4-4). Some of these dose-response curves were also subject to error as a result of a tendency for the constrictor tyramine responses to decline slightly from the peak value prior to the introduction of the next cumulative dose. These deficiencies were, however, largely overcome in the experiments utilizing nialamide *in vitro*, which were designed to estimate threshold tyramine responses.

Nialamide in vitro:

One ear artery in each of 8 rabbits was perfused for 1 hour with the monoamine oxidase inhibitor, nialamide (100 µg/ml), instead of iproniazid. Doses of tyramine were cumulated and the dose-response curves were compared, in each experiment, with those of the opposite (control) ear artery which had been perfused identically for 1 hour with Krebs bicarbonate solution. In four of the experiments, intraluminal tyramine was applied to the artery first and in the remaining 4 experiments, extraluminal tyramine was applied first. Examples of dose-response curves in control and nialamide-treated arteries from 2 rabbits, which represent the extremes (best and worst) of those obtained, are shown in

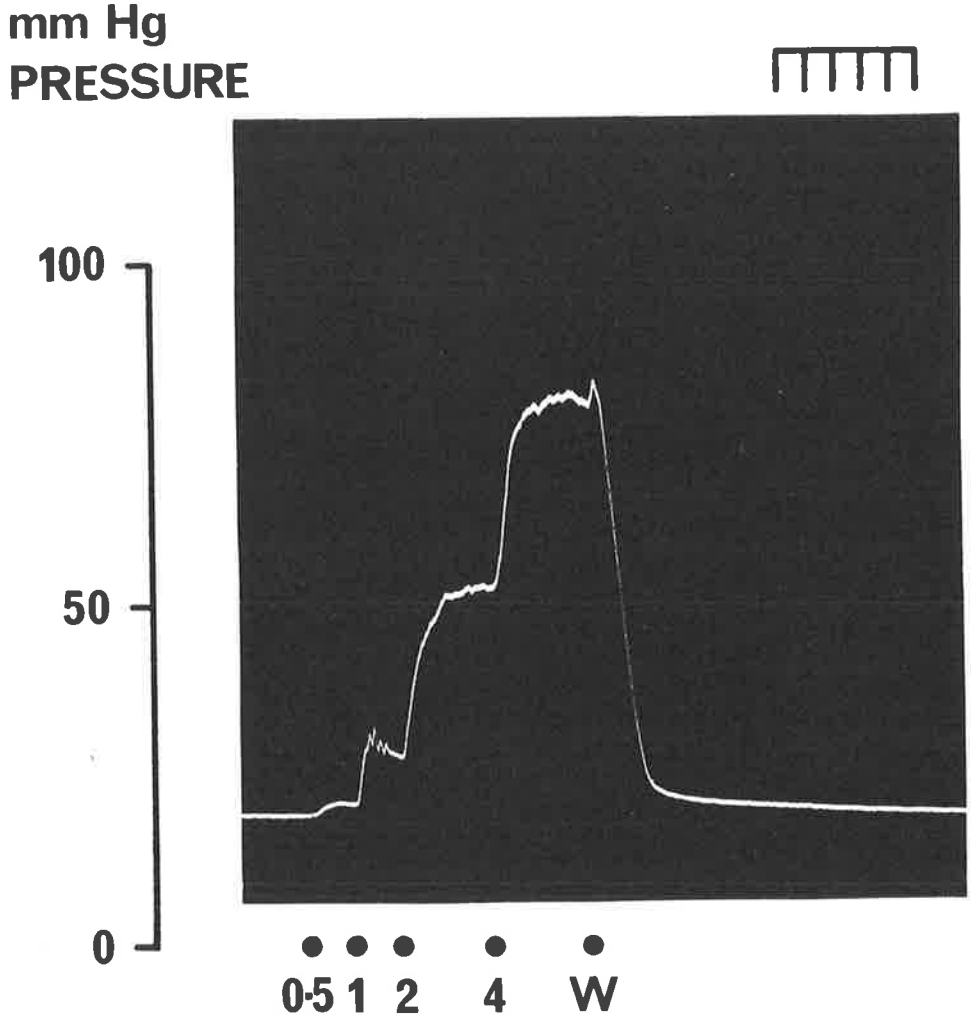


Fig. 4-4 Constrictor responses of a control (untreated) ear artery to cumulative increments of extraluminal tyramine producing final tyramine concentrations (µg/ml) as indicated by the numerals. W refers to tyramine washout.

Time scale: minutes

Fig. 4-5 and Fig. 4-6, respectively.

It will be noted in these latter 2 experiments, that despite differences in the shapes of the curves at higher levels of response, as illustrated in Fig. 4-6, the threshold concentration of extraluminal tyramine is very much smaller than that of intraluminal tyramine in each control artery. In the nialamide-treated arteries, however, the threshold concentrations of tyramine are considerably less than those in the control arteries and there is now little difference between the threshold concentrations of intraluminal and extraluminal tyramine.

That this was a general trend is indicated by comparing the threshold concentrations of tyramine in each of the 8 experiments which are represented graphically in Fig. 4-7. The mean values of the tyramine concentrations producing threshold responses in the control and nialamide-treated arteries are shown in Table 4-4. The gain in sensitivity to intraluminal and extraluminal tyramine produced by nialamide was calculated from these mean values and the results are illustrated in Table 4-5. It is seen that the effect of *in vitro* nialamide treatment was to enhance the sensitivity of the ear artery to both intraluminal and extraluminal tyramine. However, the average gain in sensitivity to intraluminal tyramine (67 fold) was much greater than that to extraluminal tyramine (7.7 fold).

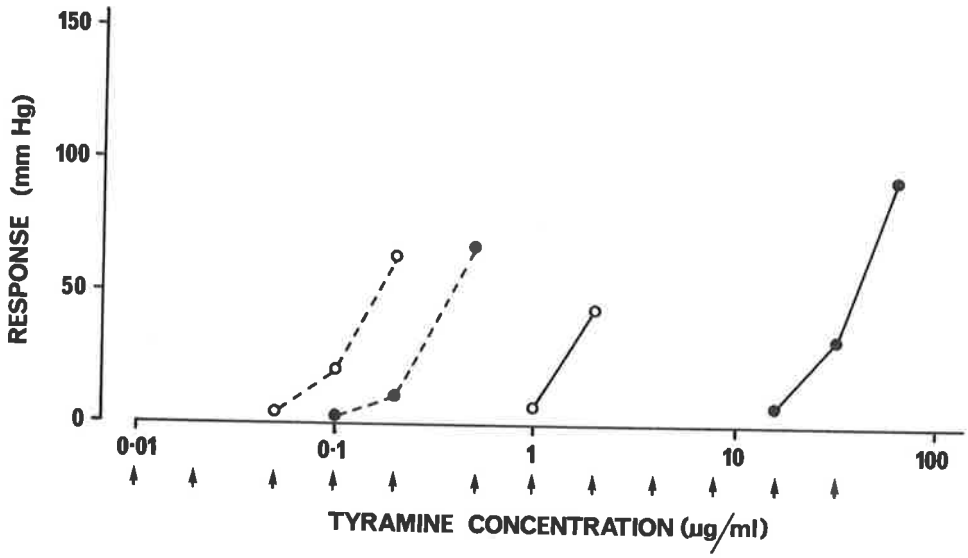


Fig. 4-5 Dose-response curves to cumulative doses of tyramine in an *in vitro* nialamide-treated ear artery (broken lines) and in its corresponding control ear artery (unbroken lines). The open symbols refer to extraluminal tyramine and the closed symbols to intraluminal tyramine. The arrows indicate the cumulative additions of tyramine in increments of 2 or 2.5 times the preceding dose in each instance. Response refers to increase in perfusion pressure above the base-line (resting level).

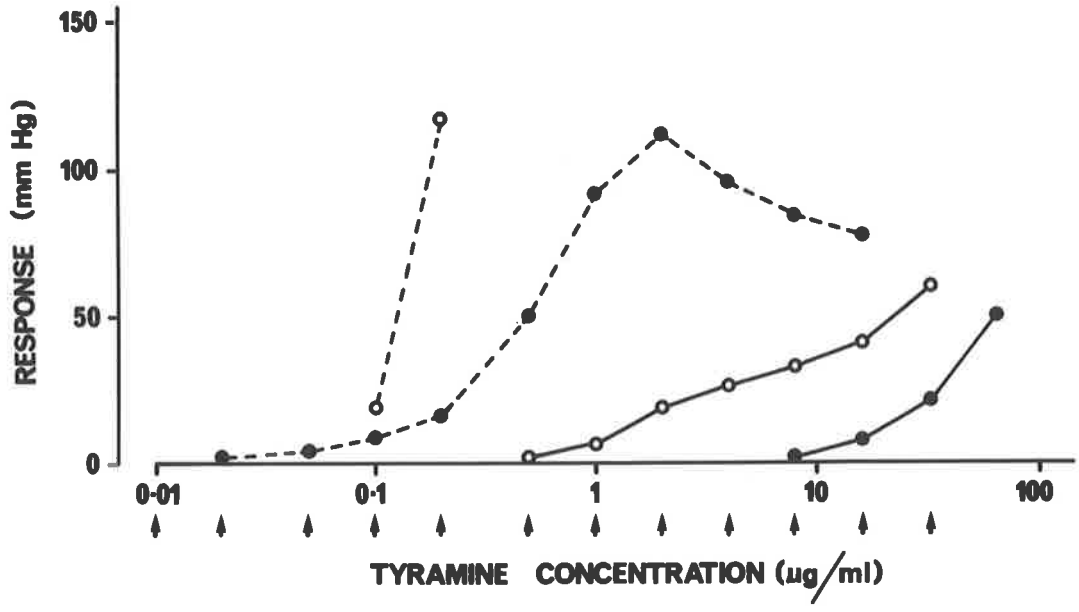
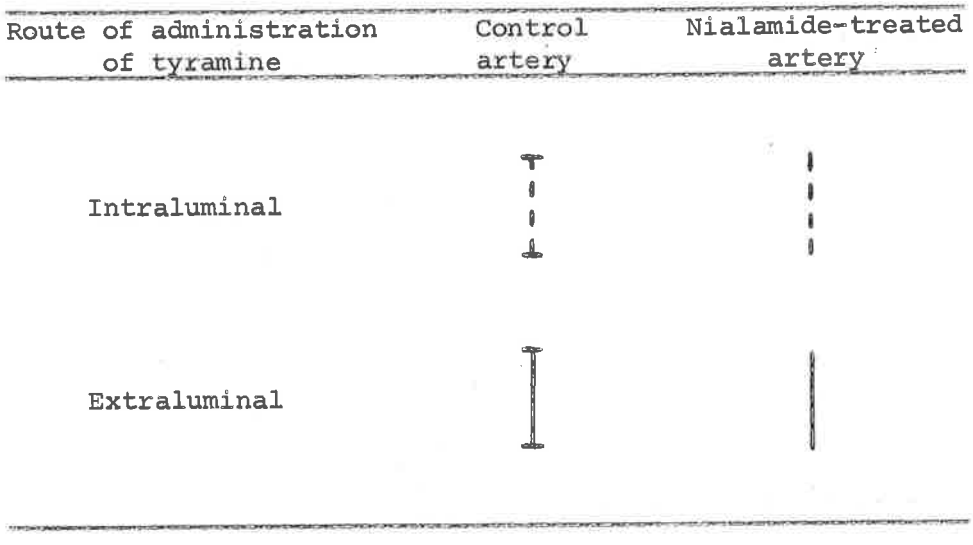


Fig. 4-6 Dose-response curves to cumulative doses of tyramine in an *in vitro* nialamide-treated ear artery (broken lines) and in its corresponding control ear artery (unbroken lines). The open symbols refer to extraluminal tyramine and the closed symbols to intraluminal tyramine. The arrows indicate the cumulative additions of tyramine in increments of 2 or 2.5 times the preceding dose in each instance. The nialamide-treated artery displays a maximal response to intraluminal tyramine, followed by a decline in sensitivity with increasing tyramine concentration. Response refers to increase in perfusion pressure above the base-line (resting level).

Fig. 4-7 Effect of monoamine oxidase inhibition on the threshold concentrations of intraluminal and extraluminal tyramine. The top of each vertical line represents the smallest cumulative tyramine concentration which first produced a response and the bottom of each line represents the immediately preceding concentration which failed to elicit a response.



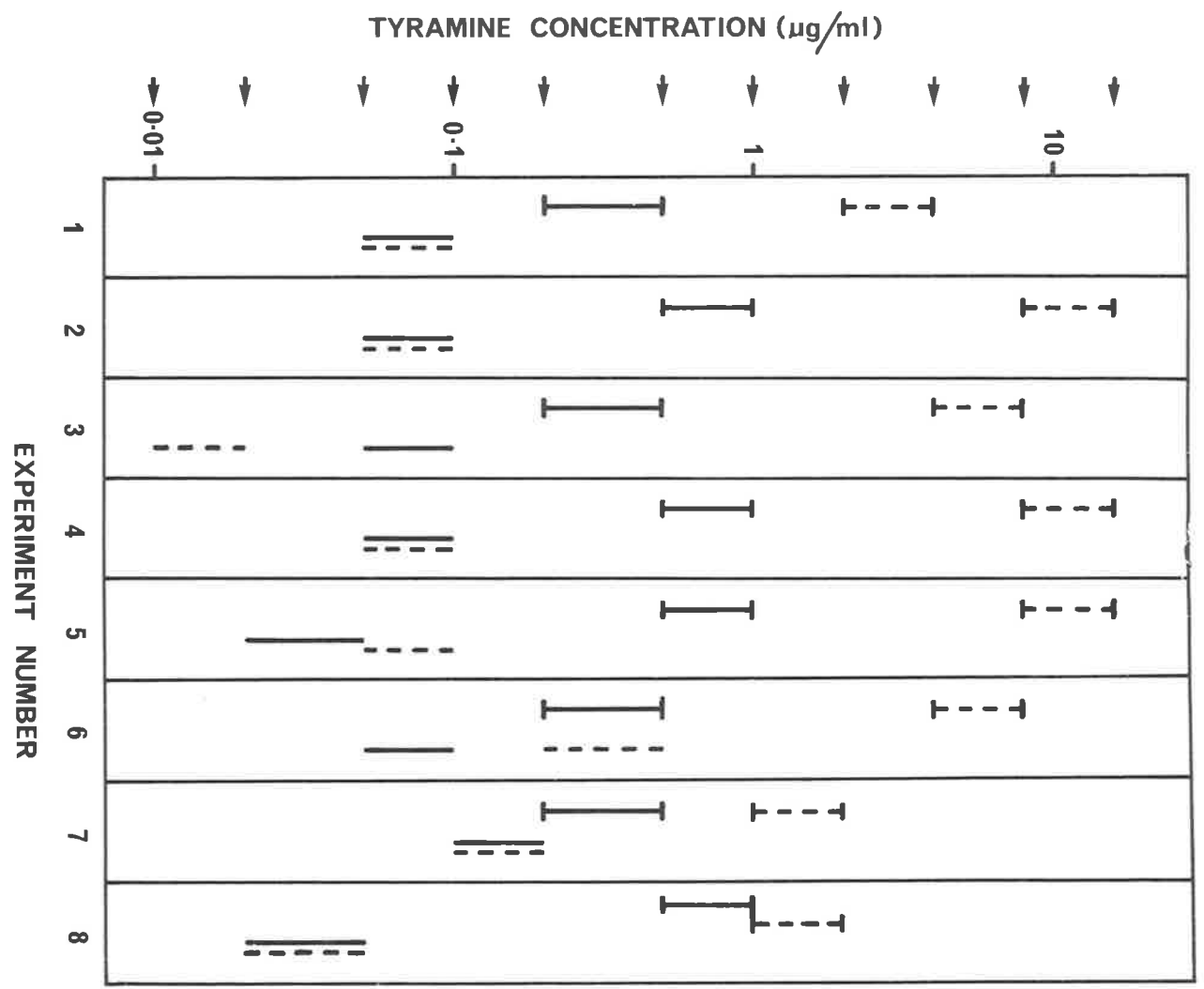

TYRAMINE CONCENTRATION (µg/ml)
0·01
0·1
1
10
EXPERIMENT NUMBER
1
2
3
4
5
6
7
8

TABLE 4-4

Equipotent concentrations of tyramine (μg/ml)

Route of administration of tyramine	Tyramine doses producing threshold responses	
	Control	Nialamide *in vitro*
Intraluminal	9.0 (11.2) (6.8)	0.15 (0.20) (0.09)
Extraluminal	0.75 (0.84) (0.66)	0.10 (0.12) (0.08)

The values shown are the arithmetic means of the threshold responses illustrated in Fig. 4-7. The figures in brackets refer respectively to mean + standard error and mean - standard error.

TABLE 4-5

Gain in sensitivity to intraluminal and extraluminal tyramine produced by nialamide in vitro, expressed as the sensitivity ratio, $\frac{Control}{Nialamide\ in\ vitro}$.

Route of administration of tyramine	Sensitivity ratio
Intraluminal	67 (106) (43)
Extraluminal	7.7 (10) (5.9)

The values shown are the geometric means of the sensitivity ratios in each of the 8 experiments illustrated in Fig. 4-7. The figures in brackets refer respectively to mean + standard error and mean - standard error. Since the means are geometric, the values of (+ standard error) and (- standard error) differ.

The threshold concentration employed in these estimates was the dose of tyramine which caused a demonstrable (greater than 2 mm) rise in perfusion pressure when the immediately preceding dose failed to elicit a response. Since all doses were increased in increments of 2 or 2.5 times the preceding dose, the above values of threshold concentration may conceivably be too high by a factor of up to, but not exceeding 2.5 times. Although this factor may affect the significance placed on the 7.7 fold reduction in the threshold concentration of extraluminal tyramine produced by nialamide treatment, it does not invalidate the conclusion that nialamide produced far greater reduction in the threshold concentration of intraluminal tyramine, since here the mean decrease was 67 fold.

Summary of data:

The results obtained using nialamide *in vitro* strongly confirmed the trends observed earlier in the iproniazid experiments. The effect of both iproniazid and nialamide treatment was to increase the sensitivity of the ear artery to both intraluminal tyramine (60 and 67 fold, respectively) and extraluminal tyramine (12 and 7.7 fold, respectively). Their action in enhancing the sensitivity to intraluminal tyramine to a much greater extent than that to extraluminal tyramine had the additional effect of reducing the difference between the intraluminal and extraluminal potencies

of tyramine which prevail in the normal (untreated) artery. Table 4-6 illustrates this latter finding with $\frac{\text{intraluminal}}{\text{extraluminal}}$ tyramine ratios derived from data in Table 4-1 and Fig. 4-7.

Cocaine effect:

The effect of cocaine on the sensitivity to tyramine in monoamine oxidase-inhibited arteries was examined in order to establish whether the response to tyramine was mediated indirectly in this situation, as it is in the normal ear artery, by the release of noradrenaline from the sympathetic nerves (de la Lande and Waterson, 1968a). The use of cocaine was based on evidence that it inhibits the major indirect component of tyramine's sympathomimetic action in the rabbit aortic strip (Furchgott, Kirpekar, Rieker and Schwab, 1963) and on supportive evidence that it has the same action on the rabbit ear artery (de la Lande and Waterson, 1968a). Professor I. S. de la Lande performed the following 3 experiments utilizing cocaine and he kindly allowed the data to be used for the purposes of this thesis.

In order to quantitate the effects of cocaine, paired ear arteries taken from 3 untreated rabbits were isolated and perfused with nialamide (100 µg/ml) for 1 hour by the double-cannulation technique as described in Chapter 2. After nialamide washout, the arteries were perfused for a further 10 minutes with Krebs bicarbonate solution. Cocaine (1 µg/ml) was then added intraluminally and

TABLE 4-6

Mean values of the sensitivity ratio $\frac{Intraluminal}{Extraluminal}$ *tyramine.*

	Control	Iproniazid *in vivo*	Nialamide *in vitro*
Data from Table 4-1	9.5 (12.5) (7.2)	2.4 (3.4) (1.7)	
Data from Fig. 4-7	9.5 (12.7) (7.2)		1.1 (1.5) (0.8)

Each result is the geometric mean of the ratios from separate experiments and the figures in brackets refer respectively to mean + standard error and mean - standard error. Since the means are geometric, the values of (+ standard error) and (- standard error) differ.

extraluminally to one artery of each pair. The threshold concentrations of intraluminal and extraluminal tyramine, in the presence and absence of cocaine, were then estimated by Method 2 as described in "Materials and Methods" in this Chapter. The results obtained are shown in Table 4-7. In each experiment, cocaine caused a 7 fold or greater reduction in sensitivity to both intraluminal and extraluminal tyramine, implying that the actions of both intraluminal and extraluminal tyramine in the monoamine oxidase-inhibited arteries were largely indirect.

Histochemistry:

At the conclusion of most experiments, the arteries were examined histochemically for the presence of monoamine oxidase by the technique of Glenner, Burtner and Brown, Jr (1957), with minor modifications. These findings, which have been described in detail in Chapter 3, have indicated that the concentrations of *in vivo* and *in vitro* iproniazid and *in vitro* nialamide employed resulted in marked inhibition of monoamine oxidase activity in the artery wall.

DISCUSSION:

The results of this study support the suggestion of de la Lande and Waterson (1968a) that monoamine oxidase in the media of the ear artery wall is one of the factors contributing to the relatively low activity of intraluminal tyramine when compared with that of extraluminal tyramine. The histochemical studies, described

TABLE 4-7

Effect of cocaine on sensitivity to intraluminal and extraluminal tyramine in nialamide treated arteries.

Threshold concentrations of tyramine (µg/ml), each expressed as a range, from the concentration without effect to the concentration which first produced a constrictor response.

Experiment Number		1	2	3
Control	Intraluminal tyramine	0.12-0.25	0.06-0.12	0.03-0.06
	Extraluminal tyramine	0.02-0.04	0.02-0.03	0.06-0.12
Cocaine (1 µg/ml)	Intraluminal tyramine	1.0 -2.0	1.0 -2.0	2.0 -4.0
	Extraluminal tyramine	0.14-0.28	0.5 -1.0	0.5 -1.0
Decrease in sensitivity to intraluminal tyramine produced by cocaine		x8	x17	x67
Decrease in sensitivity to extraluminal tyramine produced by cocaine		x7	x33	x8

in detail in Chapter 3, have confirmed, by a different technique, the original observation of Koelle and Valk, Jr (1954) that monoamine oxidase activity is present in the media of the rabbit ear artery. There was no evidence of significant activity in the region of the nerve terminals at the medial-adventitial border of the artery or of a change in distribution or intensity of activity following chronic sympathetic denervation. This latter finding was in agreement with that of Armin, Grant, Thompson and Tickner (1953) who showed that monoamine oxidase activity, measured by the rate of oxidation of tyramine, was unaffected by chronic sympathetic denervation.

The results of the perfusion experiments indicated that monoamine oxidase inhibition increased the sensitivity to intraluminal tyramine more than that to extraluminal tyramine. Thus the average increase in sensitivity to intraluminal tyramine produced by iproniazid and nialamide was 60 and 67 fold, respectively, and that to extraluminal tyramine was 12 and 7.7 fold, respectively, when estimated in terms of sensitivity ratios. This selective enhancement of intraluminal tyramine sensitivity is consistent with the presence of the enzyme between the intimal site of application of intraluminal tyramine and its presumed site of action in the sympathetic nerve terminals at the medial-adventitial border of the artery. The smaller effect of monoamine oxidase inhibition on

extraluminal tyramine sensitivity is accounted for by the absence of the enzyme between its adventitial site of application and its site of action in the nerve terminals. The assumption that the major site of action of intraluminal tyramine in the monoamine oxidase-inhibited artery is the sympathetic nerve terminals is supported by the ability of cocaine to produce an 8 fold or greater decrease in sensitivity to intraluminal tyramine. This result indicates that inhibition of monoamine oxidase enhances mainly the indirect (noradrenaline-releasing) effect of the intraluminal tyramine and may be explained in terms of an increase in the amount of tyramine reaching the nerve terminals from the intima.

Alternative explanations which may, however, have an additional bearing on the above findings are as follows:

(a) Inhibition of intraneuronal monoamine oxidase results in an accumulation of noradrenaline and absence of destruction of tyramine within the nerve terminals, the net effect being a greater release of noradrenaline from the nerve terminals.

(b) Inhibition of the medial enzyme results in a reduced rate of destruction of noradrenaline after its release.

However, neither of these alternative explanations discriminates between an intraluminal or extraluminal source of the tyramine reaching the nerve terminals and therefore cannot account for the selective potentiation of intraluminal tyramine observed in

the experiments described in this study. They may, of course, account for the 12 and 8 fold increases in sensitivity to extraluminal tyramine produced by iproniazid and nialamide, respectively. If this is the case, then their contribution to the 60 and 67 fold potentiation of intraluminal tyramine caused by iproniazid and nialamide, respectively, may also be of the order of 12 and 8 fold.

In view of the above considerations, a likely explanation for the difference between the intraluminal and extraluminal tyramine sensitivities in the normal (untreated) artery is that intraluminal tyramine is metabolized by monoamine oxidase in the media as it diffuses from the intima to the sympathetic nerve terminals at the medial-adventitial border of the artery (Fig. 4-8). It must be emphasized that in this model, those sequelae of monoamine oxidase inhibition (described previously) which are unlikely to depend upon the route of application of tyramine to the artery, have been ignored.

It is conceivable that monoamine oxidase is not the only factor contributing to the loss of tyramine as it diffuses from the intima to the sympathetic nerve terminals. Monoamine oxidase has been shown to be associated with mitochondria (Cotzias and Dole, 1951; Hawkins, 1952; Blaschko, Hagen and Hagen, 1957; Oswald and Strittmatter, 1963; Schnaitman, Erwin and Greenawalt, 1967), so that presumably tyramine must enter the smooth muscle cells of

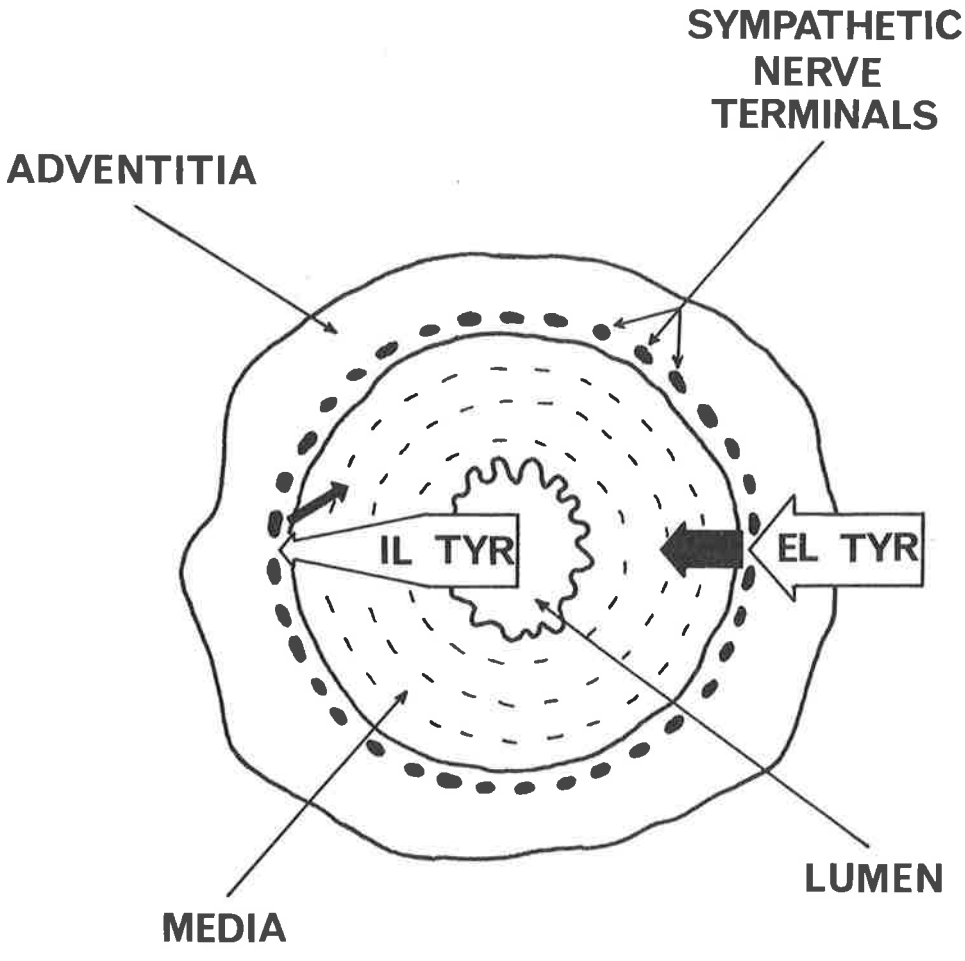


Fig. 4-8 A diagrammatic representation of the ear artery to explain the interactions of tyramine, monoamine oxidase in the media and the sympathetic nerve terminals. The open arrows refer to intraluminal (IL) and extraluminal (EL) exogenous tyramine (TYR) and the black arrows to endogenous noradrenaline liberated by the tyramine from the sympathetic nerve terminals. The thickness of the arrows reflects the concentrations of the amines.

This model was foreshadowed by de la Lande and Waterson (1968a).

the media in order to be metabolized. Entry of tyramine into the smooth muscle cells may still occur irrespective of the degree of monoamine oxidase inhibition. Nevertheless, loss of intraluminal tyramine by this mechanism must still be small compared with the loss resulting from metabolism by monoamine oxidase, as there is little difference (1.1 to 2.4 fold) between the intraluminal and extraluminal sensitivities to tyramine in the monoamine oxidase-inhibited artery. The small or negligible difference between these sensitivities tends to exclude an important role for other factors such as dilution of intraluminal tyramine by the tyramine-free extraluminal bathing solution in the vicinity of the medial-adventitial border of the artery or the presence of a permeability barrier restricting the access of intraluminal tyramine to the sympathetic nerve terminals.

SUMMARY

1. The effect of the monoamine oxidase inhibitors, iproniazid and nialamide, was to enhance the sensitivity of the isolated rabbit ear artery to both intraluminally- and extraluminally-applied tyramine.

2. The gain in sensitivity to intraluminal tyramine, however, was much greater than that to extraluminal tyramine and the difference between the intraluminal and extraluminal potencies of the drug was much less marked after monoamine oxidase inhibition.

3. Extraneuronal monoamine oxidase has been previously demonstrated histochemically throughout the media of the artery wall.

4. It is postulated that the high ratio of activity of extraluminal tyramine to intraluminal tyramine which normally prevails, is due to enzymatic destruction of the intraluminally-applied tyramine as it diffuses from the intima to the sympathetic nerve terminals at the medial-adventitial border of the artery.

C H A P T-E R 5

THE EFFECT OF MONOAMINE OXIDASE INHIBITION ON THE NORADRENALINE RESPONSE

INTRODUCTION:

The experiments described in this chapter represent an attempt to elucidate the possible significance of monoamine oxidase in the response of the rabbit ear artery to noradrenaline. In Chapter 4, evidence was presented indicating that the distribution of the enzyme in the artery wall played an important role in determining the high sensitivity of the artery to extraluminally-applied tyramine and its low sensitivity to intraluminally-applied tyramine. A previous study (de la Lande, Frewin and Waterson, 1967) showed that the position of the sympathetic nerve terminals in the wall of the rabbit ear artery was an important factor in determining differences between the vasoconstrictor responses to intraluminally-applied and extraluminally-applied noradrenaline. However, the part which monoamine oxidase may play in these latter responses is not known. The histochemical studies described in Chapter 3 have indicated that the enzyme is present throughout the media of the artery wall. However, a large body of indirect evidence has accumulated indicating that the enzyme is also present within noradrenergic nerves and nerve

terminals (Kopin and Gordon, 1962a and b, 1963; Kopin, 1964; Snyder, Fischer and Axelrod, 1965; Hamberger, Malmfors, Norberg and Sachs, 1964; Malmfors, 1965).

One of the factors prompting this present study was the possibility that intraneuronal monoamine oxidase may influence the response of the ear artery to intraluminal noradrenaline in a different fashion than that to extraluminal noradrenaline and accordingly the effect of monoamine oxidase inhibition upon the response of the artery to intraluminal and extraluminal noradrenaline was estimated. In other tissues, intraneuronal monoamine oxidase has been assigned an important role in the response of the isolated guinea-pig atrium to prolonged contact with noradrenaline (Furchgott and Sanchez Garcia, 1968), whereas the data of Kalsner and Nickerson (1969) have indicated that extraneuronal monoamine oxidase and catechol-O-methyl transferase appeared to be of greater importance in the response of the rabbit aortic strip to prolonged exposure to noradrenaline in high concentration.

In the present study, the effect of monoamine oxidase inhibition on the noradrenaline response in the rabbit ear artery has been examined with particular reference to the kinetics of the response. Procedures which modify this effect of enzyme inhibition by disrupting or inhibiting uptake and binding of noradrenaline by the sympathetic nerves and nerve terminals, have also been applied. Such techniques have included destruction of the nerves by chronic

sympathetic denervation (de la Lande and Rand, 1965; de la Lande, Frewin and Waterson, 1967), inhibition of noradrenaline uptake by cocaine (Macmillan, 1959; Whitby, Hertting and Axelrod, 1960; Muscholl, 1961; Hillarp and Malmfors, 1964; de la Lande and Waterson, 1967; de la Lande, Frewin and Waterson, 1967) and depletion of the noradrenaline stores by reserpine which interferes with the retention of noradrenaline in the noradrenergic storage granules (Kopin and Gordon, 1962b; Iversen, Glowinski and Axelrod, 1965; Glowinski, Iversen and Axelrod, 1966).

In their studies on the isolated guinea-pig atrium, Furchgott and Sanchez Garcia (1968) showed that provided sensitization was judged solely on the magnitude of the response height during brief (less than 5 minute) exposures to noradrenaline, monoamine oxidase inhibition was without significant effect on the sensitivity of the preparation to noradrenaline. Initial experiments with brief exposures of the rabbit ear artery to noradrenaline showed a similar absence of a significant increase in sensitivity following monoamine oxidase inhibition. Accordingly, these findings were further investigated in an attempt to define their relationship to the results obtained with more prolonged exposures of the ear artery to noradrenaline.

In the course of the experiments investigating the kinetics of the noradrenaline response, the findings (as will be described in

Results) indicated that intraluminally-applied noradrenaline did not produce the same changes as extraluminally-applied noradrenaline. This posed the question that intraluminal noradrenaline may not reach the sympathetic nerve terminals at the medial-adventitial border of the artery in contradistinction to easy access of extraluminal noradrenaline. In order to further elucidate this problem, arteries taken from reserpine-pretreated rabbits were examined by the histochemical fluorescence technique as described in Chapter 2, after prolonged (30 minute) application of intraluminal or extraluminal noradrenaline. This histochemical procedure was thought likely to add value to the general interpretation of the physiological findings.

MATERIALS AND METHODS:

Perfusion of the ear artery:

Following excision, a segment of the central artery of the rabbit ear was cannulated and perfused by the double-cannulation technique described in Chapter 2. Double cannulation of the artery enabled comparisons to be made between vasoconstrictor responses to noradrenaline added extraluminally to the extraluminal Krebs bicarbonate solution and the responses to noradrenaline applied intraluminally by addition to the reservoir of intraluminal Krebs bicarbonate solution.

Application of noradrenaline to the artery:

In the experiments in which sensitivity changes were

recorded, as measured by the magnitude of the noradrenaline response, a period of contact which was sufficient to establish the initial plateau of the response (usually less than 4 to 5 minutes) was employed. However, a longer period of contact of 30 minutes was used routinely in experiments where the kinetics of the response (the shape of the response and the recovery from the response) were being studied. In a number of experiments, it was necessary to measure the recovery times of noradrenaline responses in arteries which had been exposed to identical concentrations (0.5 µg/ml) of noradrenaline, under conditions where the sensitivity of the arteries to noradrenaline differed widely. This concentration had to be sufficiently high to produce an adequate response (of the order of 100 to 120 mm of mercury) in the least sensitive artery. This raised the problem, however, that the perfusion pressure in the more sensitive artery usually rose well above the limits for satisfactory recording (approximately 180 mm of mercury).

It was thus necessary to lessen the possibility of mechanical damage to arteries resulting from perfusion at high pressure for long periods of time. This was done by reducing the rate of flow during intraluminal noradrenaline perfusion to 1 ml/min and the flow was stopped entirely during exposure to extraluminal noradrenaline. In such experiments, kymographic recordings were not made during these 30 minute noradrenaline exposures, but were

resumed just prior to washout of the drug. Normal flow was resumed simultaneously with the washout of the noradrenaline. The perfusion pressure remained high immediately after washout and flow was stopped periodically until the perfusion pressure had fallen to approximately 180 mm of mercury. However, the period of time in which intermittent flow was required was rarely greater than 2 minutes and normal flow was maintained for the remainder of the recovery period. The conditions of slow (1 ml/min) intraluminal noradrenaline perfusion are referred to in Results as "minimum flow". Further details of the method by which this was achieved are presented in the Appendix, page A-2.

Inhibition of monoamine oxidase in vitro:

In the experiments in which the kinetics of the noradrenaline response were being investigated as described above, two arteries, one from each ear of the same rabbit in each case, were excised. One artery was perfused with the monoamine oxidase inhibitor, nialamide (100 µg/ml), by adding the drug to the intraluminal perfusion solution and also to the extraluminal solution bathing the artery. Under these conditions, nialamide has been shown in Chapter 3 to effectively inhibit monoamine oxidase. These solutions were replaced with drug-free media after 1 hour and the artery was then perfused for a further 10 minutes prior to examination of the noradrenaline responses. The opposite ear artery was

used as a control in that it was not perfused with the inhibitor but was perfused with drug-free Krebs bicarbonate solution for the same period of time (1 hour). Following the 10 minute washout period, the responses to 30 minute applications of intraluminal and extraluminal noradrenaline, respectively, were examined.

Estimation of vascular sensitivity change:

Monoamine oxidase inhibition was achieved in the above manner in the experiments designed to measure its effect on the sensitivity of the ear artery to noradrenaline. Responses to both intraluminally-applied and extraluminally-applied noradrenaline were initially elicited before exposure of 7 arteries to nialamide (100 µg/ml) which was applied for 1 hour both by intraluminal perfusion and by addition to the extraluminal bathing solution. Following washout with drug-free Krebs bicarbonate solution for 10 minutes, noradrenaline responses were obtained as before. In order to quantitate any non-specific increase in sensitivity of the artery occurring during the period of nialamide perfusion, 7 control arteries were treated identically except that Krebs bicarbonate solution was applied for 1 hour instead of nialamide. The arteries used in these experiments were removed from different animals and were thus non-paired.

Constrictor responses to short (2 to 5 minute) applications of noradrenaline were recorded according to the "maximum" rise in

perfusion pressure attained during or just after administration of the drug. Measurement of this "maximum response" is further discussed in Results. Such responses were recorded in duplicate at two concentration levels of noradrenaline before and after application of nialamide or Krebs bicarbonate solution. Dose-response curves were established as described in Chapter 2 and sensitivity ratios were determined by measurement of the respective intraluminal and extraluminal doses of noradrenaline producing responses of 60 mm of mercury.

Noradrenaline fluorescence histochemistry:

In 9 experiments, the ear arteries from each of 9 rabbits which had been pretreated with reserpine to deplete the noradrenaline stores, were removed and two segments of one ear artery in each animal were double-cannulated and perfused by the method described in Chapter 2. A segment of the opposite ear artery in each case was single-cannulated and all three arteries were then treated with nialamide (100 µg/ml) for 1 hour as described previously. After perfusion with Krebs bicarbonate solution for 20 minutes, noradrenaline (0.5 µg/ml) was applied extraluminally to one of the double-cannulated arteries and intraluminally to the other for 30 minutes, by the same technique as described previously for the physiological experiments. Ten minutes after washout of the noradrenaline, the arteries were taken for histochemical analysis

by the method of Falck (1962), as modified by Waterson and Smale (1962) and as described in Chapter 2. The single-cannulated artery which was similarly examined, served as a non-fluorescent control, in that it was perfused only with Krebs bicarbonate solution and had no contact with noradrenaline.

Reserpine pretreatment:

Thirteen rabbits received reserpine (2.5 mg/Kg body weight) by intraperitoneal injection 24 hours prior to removal of the ear arteries.

Chronic sympathetic denervation:

The central artery of the left ear was denervated in each of 7 rabbits by prior removal of the respective superior cervical ganglion 6 to 13 days previously. The procedure employed was that of de la Lande and Rand (1965) and both their method and the tests of its effectiveness have been described in detail in Chapter 2.

Metabolic oxidation of applied noradrenaline:

In the experiments in which the arteries were exposed to noradrenaline for 30 minute periods, metabolic oxidation of the drug became a potential problem. In order to prevent this occurrence, ascorbic acid solution was added respectively to the intraluminal noradrenaline perfusion solution and to the extraluminal noradrenaline bathing solution so that the final concentration of ascorbic acid was 1 in 20,000.

RESULTS:

Kinetics of the response to prolonged noradrenaline exposure:

In 5 untreated arteries, the responses to intraluminal and extraluminal noradrenaline were similar in shape and time course. Each response comprised an increase in perfusion pressure which was rapid in onset and well-sustained at a steady (plateau) level for the 30 minute period of exposure to noradrenaline. Following washout of noradrenaline, the perfusion pressure returned to its resting level (Fig. 5-1). In 11 nialamide-treated arteries, the response to intraluminal noradrenaline was similar to that in untreated arteries, whereas the response to extraluminal noradrenaline differed in two respects. Firstly, in nine of the 11 arteries, the initial rapid increase in perfusion pressure was followed by a further progressive rise in pressure throughout the period of exposure to noradrenaline. This phenomenon will be described as the secondary response. Secondly, in all eleven of the arteries, following washout of the noradrenaline, the return to resting perfusion pressure was extremely slow (delayed recovery) and was usually longer than 60 minutes. These differences are also illustrated in Fig. 5-1. It will be noted that the decline in perfusion pressure after noradrenaline washout was very rapid initially, but then became much more gradual. Often there was a rise in perfusion pressure between the rapid and slow phases of the decline.

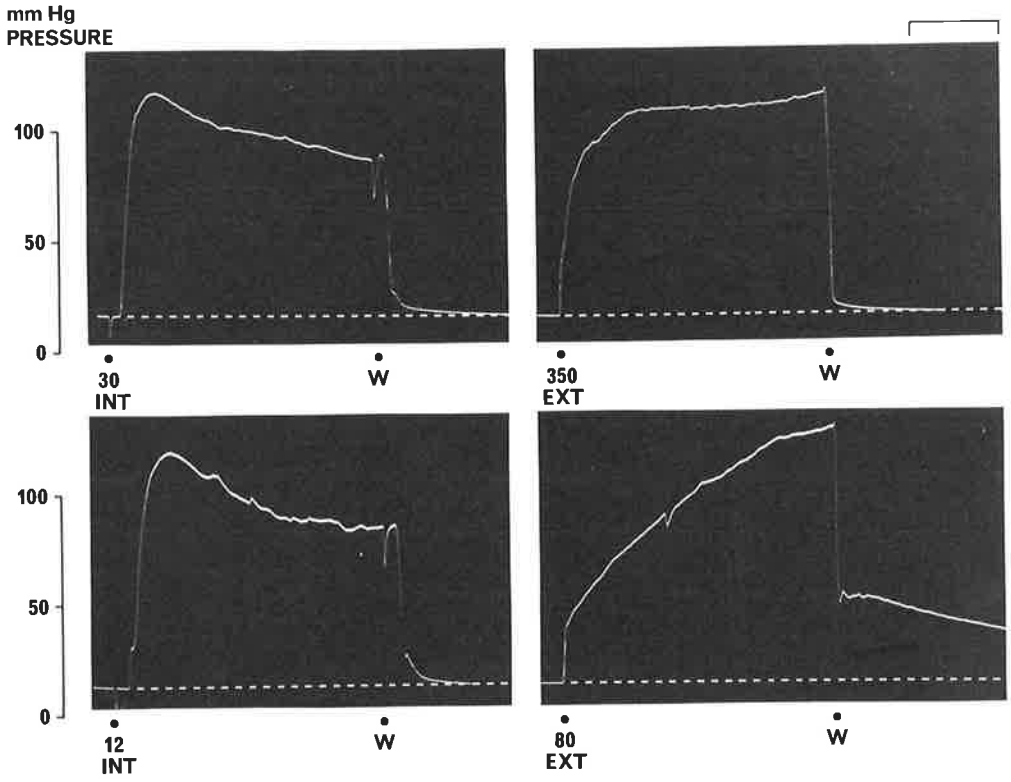


Fig. 5-1 Responses of an untreated artery (top panel) and the nialamide-treated artery from the opposite ear of the same rabbit (bottom panel) to prolonged (30 minute) exposures to intraluminal (INT) and extraluminal (EXT) noradrenaline. The numerals shown indicate dosage (ng/ml) of noradrenaline and W refers to standard noradrenaline washout followed by the recovery phase of each response. The secondary response and delayed recovery are only seen with extraluminal noradrenaline in the nialamide-treated artery. The base-line is represented by the horizontal interrupted white line in each case. (Base-line = resting level.)

Time scale: 10 minutes

Mechanism of the secondary response:

de la Lande, Frewin and Waterson (1967) showed previously that the magnitude of the response to extraluminal noradrenaline was greatly influenced by the sympathetic nerve terminals situated at the medial-adventitial border of the artery. The following experiments were performed in order to determine whether the sympathetic nerve terminals and their uptake of noradrenaline played a role in the secondary response.

In each of 4 experiments, the responses to extraluminal noradrenaline of a denervated artery from one ear were compared with those of the innervated artery from the opposite ear of the same animal in each case. Each artery was perfused with nialamide (100 µg/ml) for 1 hour, as described in Methods. None of the denervated arteries (an example of which is shown in Fig. 5-2) showed either the secondary response or delayed recovery following noradrenaline washout. In three of these experiments, the responses to intraluminal noradrenaline were also examined. These were indistinguishable in shape and time course from the responses to intraluminal noradrenaline in the innervated arteries, in that they failed to display either the secondary response or delayed recovery following washout of noradrenaline.

Cocaine has been shown to block uptake of noradrenaline by the sympathetic nerve terminals in the ear artery (de la Lande

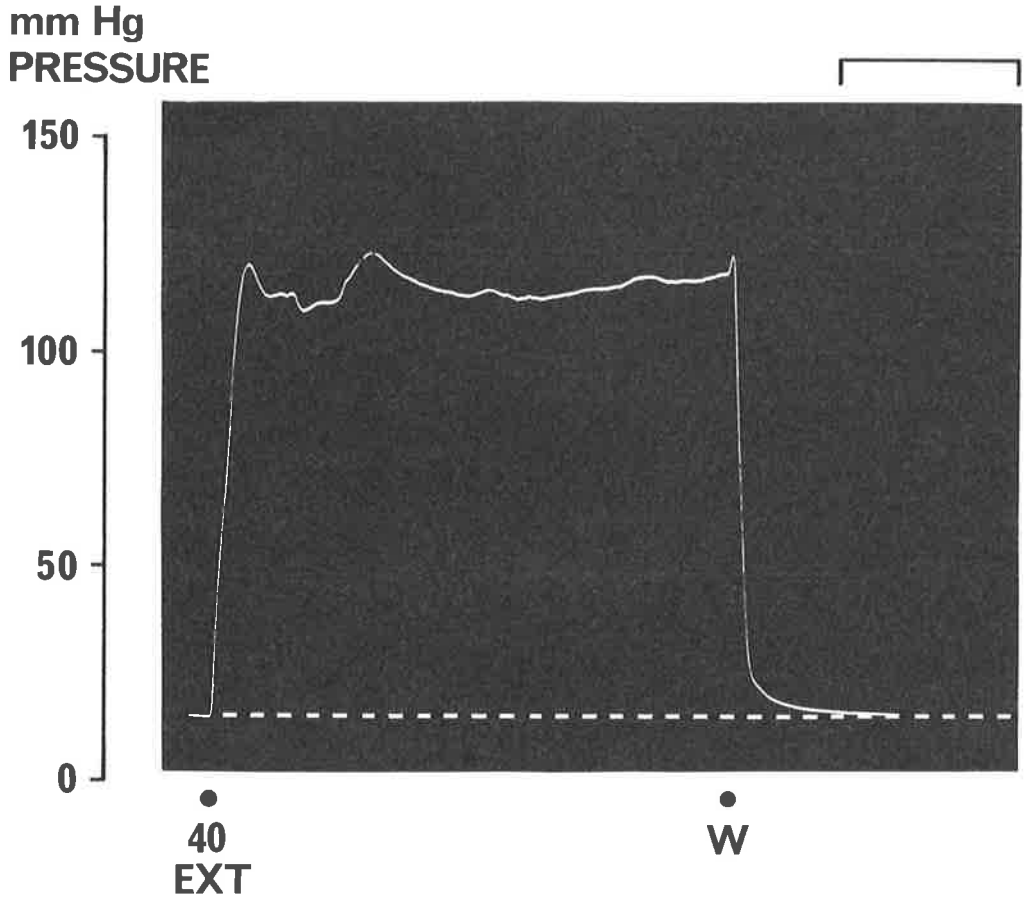


Fig. 5-2 Response of a denervated nialamide-treated artery to prolonged (30 minute) exposure to extraluminal (EXT) noradrenaline in the dosage (ng/ml) indicated by the numeral. W refers to standard noradrenaline washout followed by the recovery phase of the response. The base-line is represented by the horizontal interrupted white line.

Time scale: 10 minutes

and Waterson, 1967; de la Lande, Frewin and Waterson, 1967) and on this account, it was of interest to determine the effect of cocaine on the secondary response and on delayed recovery. Accordingly, in each of 3 experiments, 2 innervated arteries (one from each ear of the same animal) were perfused with nialamide (100 µg/ml) for 1 hour, after which cocaine (1 µg/ml) was added to both the intraluminal and extraluminal solutions of one of the arteries prior to and during the entire period of application of the extraluminal noradrenaline. The cocaine was washed out simultaneously with the noradrenaline. As in the case of the denervated arteries, responses of comparable size in the cocaine-treated arteries failed to show either the secondary response with extraluminal noradrenaline or delayed recovery following noradrenaline washout (Fig. 5-3).

From these results, it would appear that the secondary response seen with extraluminal noradrenaline in nialamide-treated arteries depended upon (1) the presence of the sympathetic nerve terminals in the artery wall and (2) uptake of noradrenaline by these nerve terminals. However, this interpretation is complicated by the fact that cocaine treatment and chronic sympathetic denervation each caused a marked increase in sensitivity to extraluminal noradrenaline, which approached the high degree of sensitivity normally displayed by the artery to intraluminal noradrenaline. It might be argued, therefore, that the failure to observe the

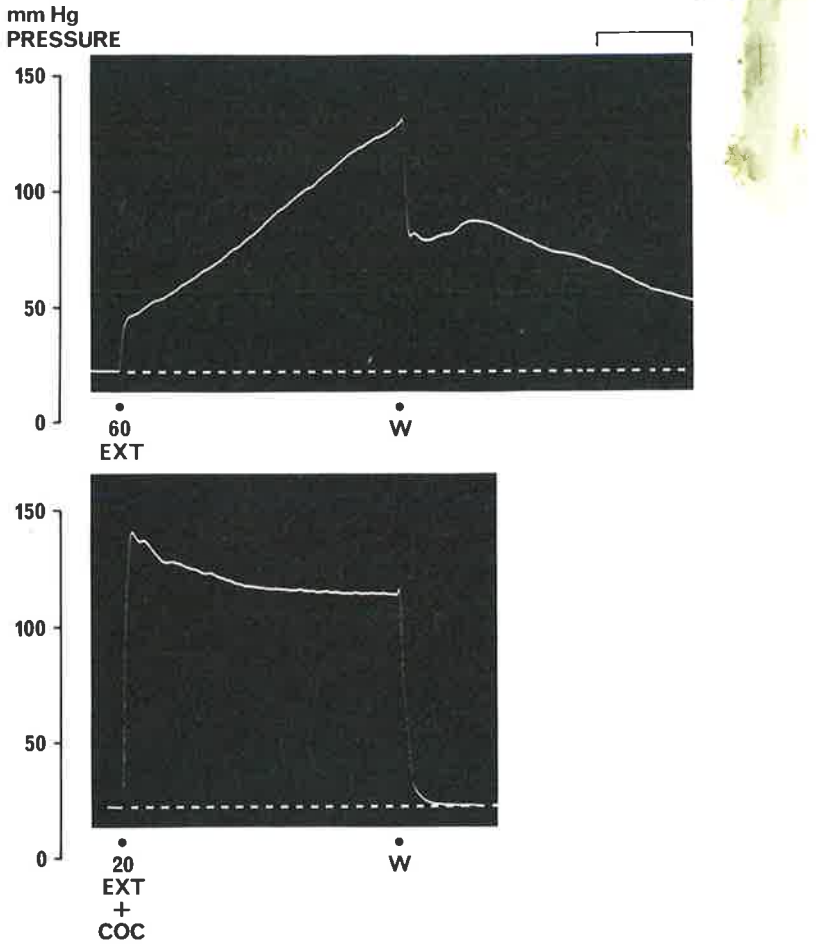


Fig. 5-3 Responses of a nialamide-treated artery to prolonged (30 minute) exposures to extraluminal (EXT) noradrenaline. The top panel illustrates the secondary response and delayed recovery and the bottom panel shows the absence of both phenomena when cocaine (1 µg/ml) was added extraluminally at the same time as the noradrenaline dose. The numerals shown indicate dosage (ng/ml) of noradrenaline and W refers to standard washout of the extraluminal drugs followed by the recovery phase of each response. The baseline is represented by the horizontal interrupted white line in each case.

Time scale: 10 minutes

secondary response and delayed recovery with extraluminal application of noradrenaline to nialamide-treated arteries which had also been cocaine-treated or denervated, reflects the relatively low concentrations of noradrenaline to which these arteries were exposed. A similar argument may be applied to the absence of the secondary response or delayed recovery on exposure of nialamide-treated arteries to intraluminal noradrenaline since the equipotent concentrations of intraluminal noradrenaline were much less than those of extraluminal noradrenaline (as seen, for example, in Fig. 5-1).

In order to overcome these objections, it was necessary to employ a standard concentration of noradrenaline in all of the different experimental situations. It was not possible to do this in the preceding experiments comparing the shapes of responses, as a concentration of noradrenaline producing an adequate increase in perfusion pressure (a response of the order of 100 to 120 mm of mercury) under the conditions where the artery was least sensitive (extraluminal noradrenaline application to an untreated innervated artery) produced responses which were above the limit of effective recording pressure (180 mm of mercury) in arteries which displayed marked sensitivity (extraluminal noradrenaline application to nialamide-treated arteries which had either been denervated or treated with cocaine). This difficulty was overcome, however, in the following experiments which were designed only to estimate the

times of recovery from noradrenaline responses in paired arteries under the same conditions of denervation and cocaine treatment.

Mechanism of delayed recovery:

In these experiments, a standard concentration of noradrenaline (0.5 µg/ml) was used, which was sufficiently high to ensure an adequate response (of the order of 100 to 120 mm of mercury) in the least sensitive artery. In order to avoid the resultant very large responses to noradrenaline under the conditions of marked arterial sensitivity, minimal flow conditions were employed with intraluminal noradrenaline and perfusion was stopped entirely during exposure to extraluminal noradrenaline.

In the estimation of the time of recovery of the noradrenaline response, no attempt was made to determine the precise point of return to the resting level as often the rate of decline was very slow so that the response approached the resting level in a very gradual manner. This problem was overcome by measuring the recovery time after noradrenaline washout in terms of the time required for the perfusion pressure to return to a fixed pressure (5 mm of mercury) above the resting level.

Hence, rates of recovery from intraluminal and extraluminal noradrenaline (0.5 µg/ml) were compared in (1) paired untreated and nialamide-treated innervated arteries (6 experiments), (2) paired nialamide-treated innervated and denervated arteries

(3 experiments) and (3) the presence and absence of cocaine (1 µg/ml), applied simultaneously with the noradrenaline (3 experiments). The results are summarized in Tables 5-1 and 5-2 and typical comparisons are shown, respectively, in Figs. 5-4, 5-5 and 5-6. It will be noted that delayed recovery only occurred in the monoamine oxidase-inhibited arteries which possessed intact sympathetic innervation and which had been exposed to extraluminal noradrenaline. It did not occur in untreated innervated arteries or in nialamide-treated arteries which had been either denervated or treated with cocaine prior to and during noradrenaline application. Delayed recovery was not seen following intraluminal noradrenaline application in any of these experimental situations.

These results therefore suggested that delayed recovery, like the secondary response, also depended upon monoamine oxidase inhibition in the presence of intact sympathetic nerve terminals and upon uptake of extraluminal noradrenaline by these terminals.

Effect of reserpine on delayed recovery:

The effect of reserpine was examined in view of its action in depleting tissues of noradrenaline by interfering with the retention of noradrenaline by the sympathetic intraneuronal storage granules. In 4 experiments, arteries were taken from rabbits which had been given reserpine (2.5 mg/Kg) by intraperitoneal injection 24 hours previously. The arteries were perfused with

TABLE 5-1

Recovery times (minutes) after exposure to intraluminal (Int) and extraluminal (Ext) noradrenaline (0.5 μg/ml) for 30 minutes - effect of nialamide treatment.

	Control artery (no nialamide treatment)		Nialamide-treated artery	
Noradrenaline application	Int	Ext	Int	Ext
Experiment Number 1	2; 2	1	3; 5	> 80
2	5; 8	1	5	>110
3	1; 1*	1	4; 5*; 4*	> 60
4	2; 2*; 1*	3; 2	4; 1*	72
5	3*	2		> 90
6	3*	1	6*; 3	>100

The recovery time after noradrenaline washout was measured in terms of the time required for the perfusion pressure to return to a fixed pressure (5 mm of mercury) above the resting level (base-line). The asterisk refers to the recovery time following simultaneous exposure of the artery to intraluminal noradrenaline and extraluminal paraffin - discussed subsequently in the text. These 6 experiments were performed on paired arteries in each case.

TABLE 5-2

Recovery times (minutes) after exposure to intraluminal (Int) and extraluminal (Ext) noradrenaline (0.5 µg/ml) for 30 minutes - effect of chronic sympathetic denervation, cocaine treatment and reserpine pretreatment on nialamide-treated arteries.

Nialamide-treated arteries		No additional treatment		Additional treatment	
Noradrenaline application		Int	Ext	Int	Ext
Additional treatment					
Denervation	Exp. No. 1	2	32	4	4
	2	1; 1*	>50	3	2
	3	4	>68	3	4
Cocaine (1 µg/ml) throughout	4	2	113	3	5
	5	3	34	5	3
	6	5	32	4	3
Reserpine pretreatment	7			2; 1; 4	> 67
	8			2	>100
	9			1	> 85
	10			5	> 85

Recovery times were measured as described in Table 5-1. The asterisk refers to the recovery time following simultaneous exposure of the artery to intraluminal noradrenaline and extraluminal paraffin. The first 6 experiments were performed on paired arteries.

Fig. 5-4 Paired untreated and nialamide-treated innervated arteries from the same rabbit showing the recovery phase of the responses to prolonged (30 minute) exposures to intraluminal (INT) and extraluminal (EXT) noradrenaline (0.5 µg/ml). The recovery phases shown in the top panel were those seen in the untreated (control) artery and the bottom panel illustrates recoveries in the opposite ear artery which had been treated with nialamide (100 µg/ml) for 1 hour. W refers to standard noradrenaline washout. Delayed recovery is only seen in the nialamide-treated artery which had been exposed to extraluminal noradrenaline. The base-line (resting level) is represented by the horizontal white line in each case.

Time scale: 10 minutes

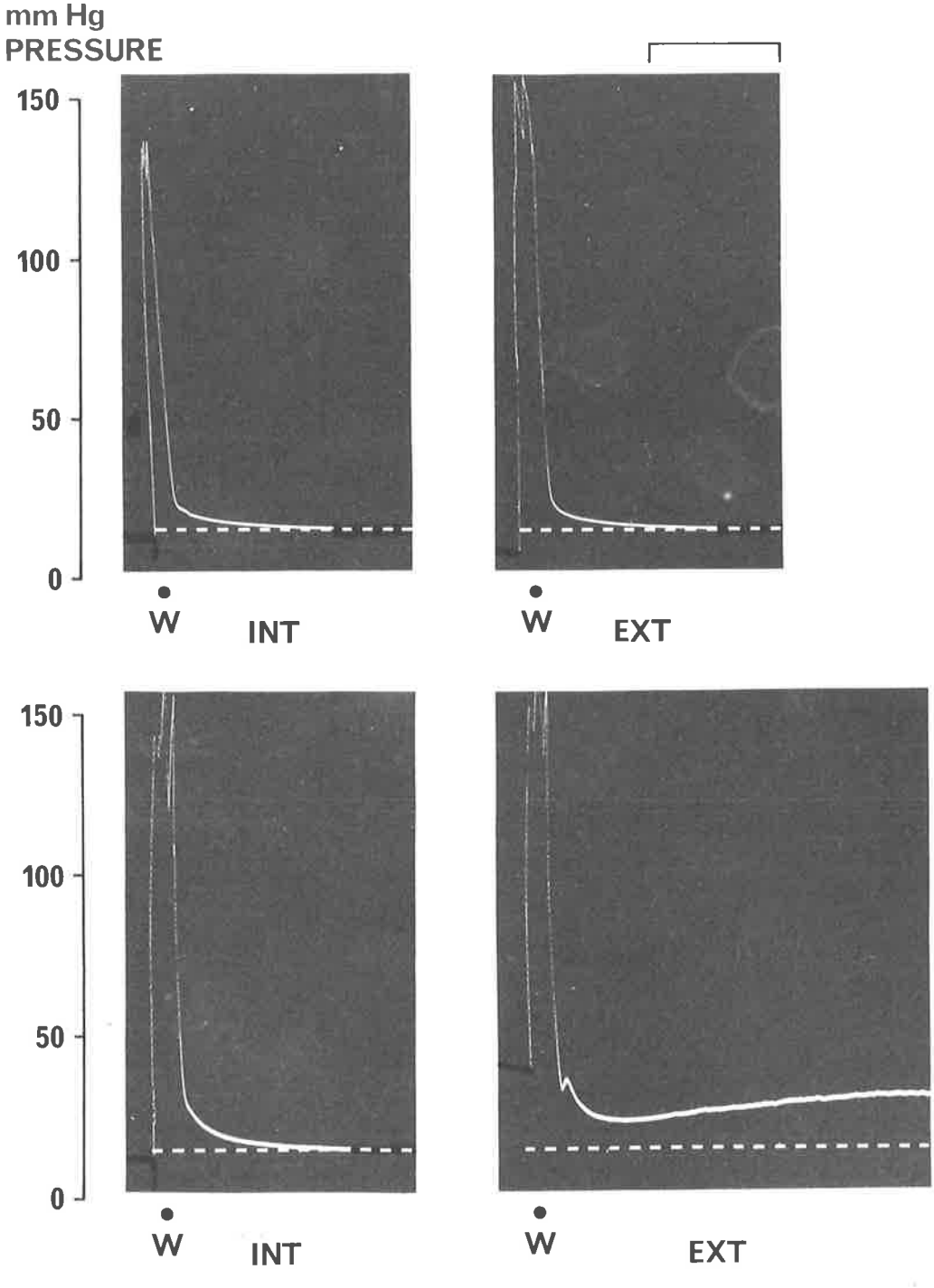

mm Hg
PRESSURE
150
100
50
0
W
INT
W
EXT
150
100
50
0
W
INT
W
EXT

Fig. 5-5 Paired nialamide-treated arteries from the same animal showing the recovery phase of the responses to prolonged (30 minute) exposures to intraluminal (INT) and extraluminal (EXT) noradrenaline (0.5 µg/ml). The recovery phases shown in the top panel were those seen in a control innervated artery and the bottom panel illustrates the effects of sympathetic denervation of the opposite ear 8 days previously. W refers to standard noradrenaline washout. Delayed recovery only occurred in the innervated nialamide-treated artery which had been exposed to extraluminal noradrenaline. The base-line is represented by the horizontal interrupted white line in each case.

Time scale: 10 minutes

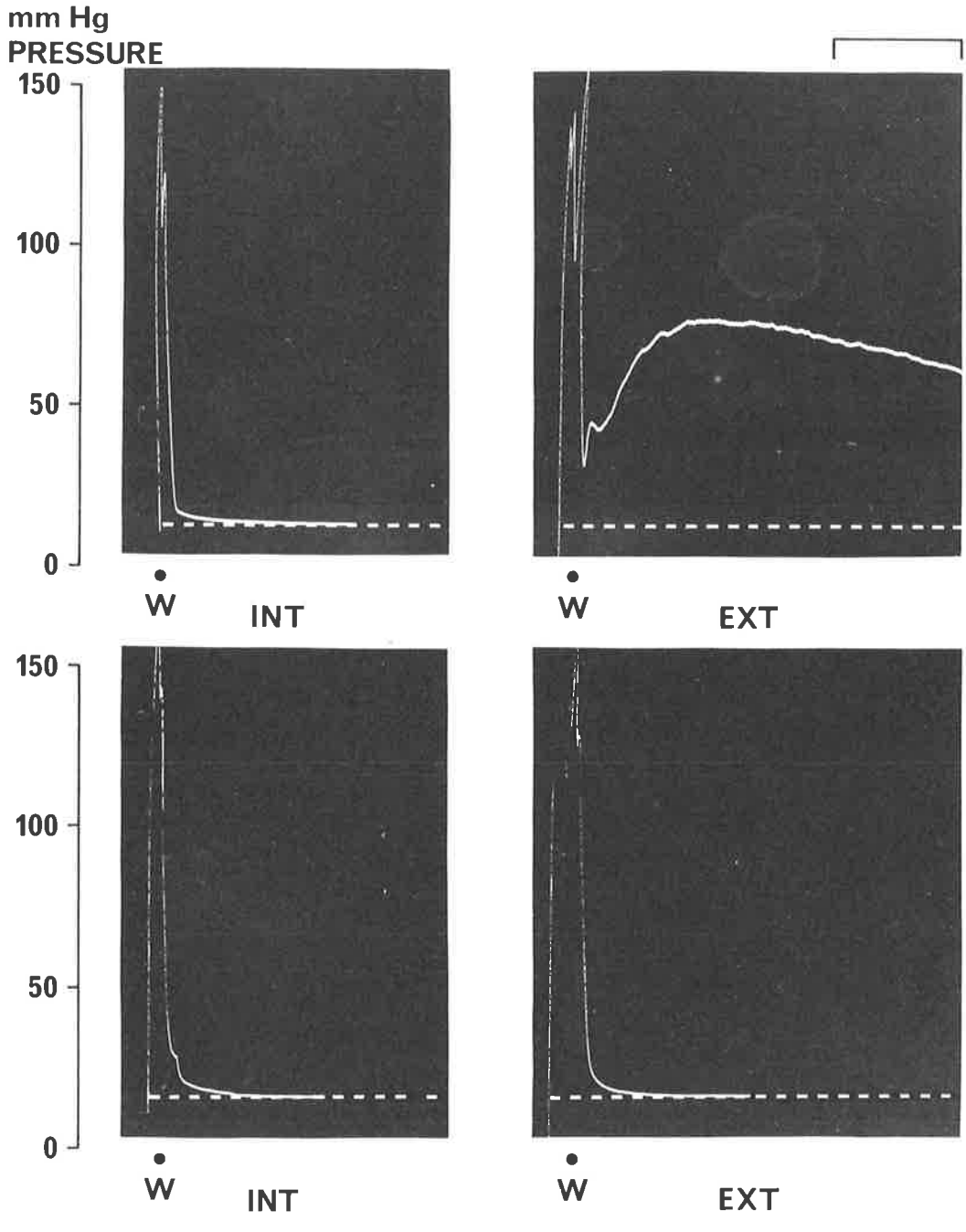
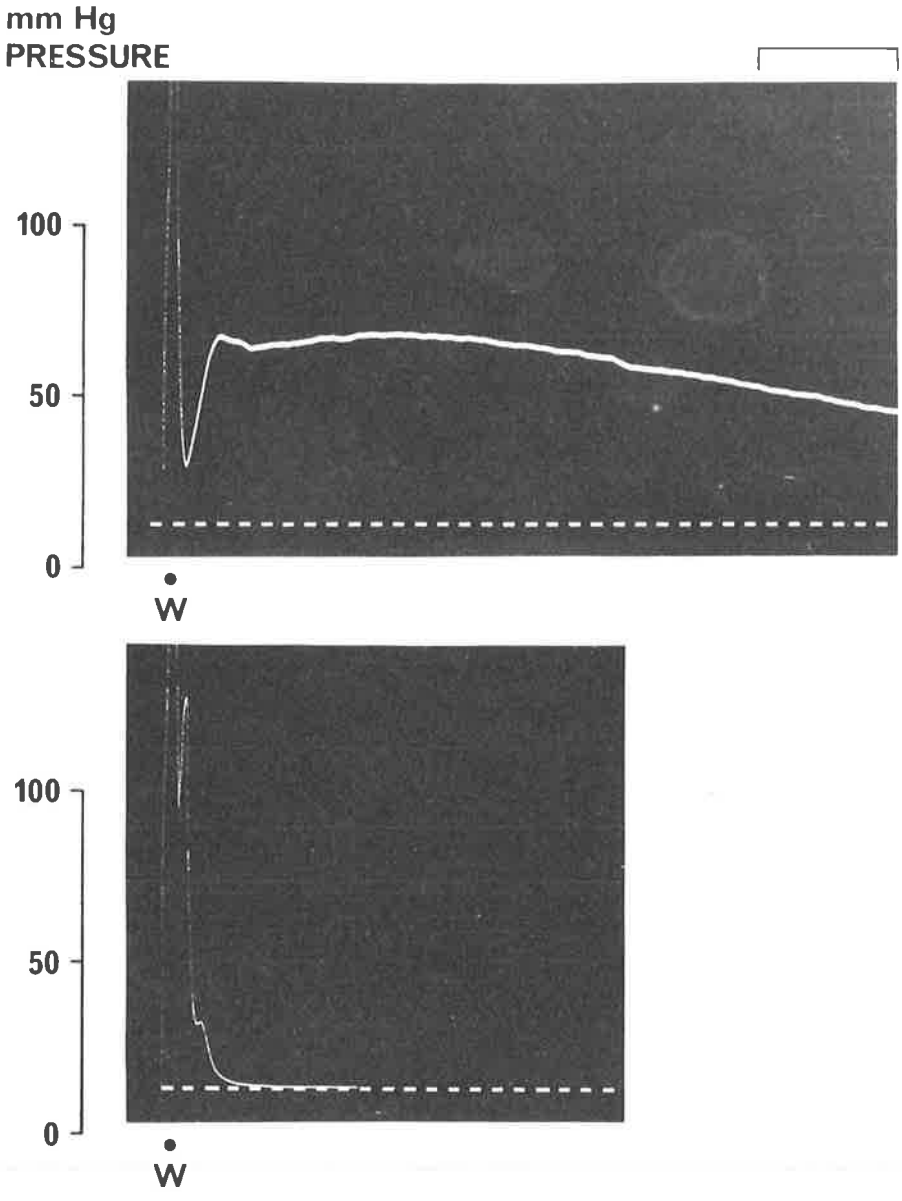

mm Hg
PRESSURE
150
100
50
0
W
INT
W
EXT
150
100
50
0
W
INT
W
EXT

Fig. 5-6 Recovery phase of the response in a nialamide-treated artery following prolonged (30 minute) exposure to extraluminal noradrenaline (0.5 µg/ml). The top panel illustrates delayed recovery and the bottom panel shows the absence of delayed recovery in the same artery when cocaine (1 µg/ml) was added extraluminally at the same time as the noradrenaline dose. W refers to standard washout of the extraluminal drugs. The baseline is represented by the horizontal interrupted white line.

Time scale: 10 minutes


mm Hg
PRESSURE
100
50
0
W
100
50
0
W

nialamide (100 µg/ml) as described in Methods and the responses to prolonged (30 minute) applications of noradrenaline were examined. Reference again to Table 5-2 shows that intraluminal noradrenaline still failed to display delayed recovery following washout. However, the delay in recovery from extraluminal noradrenaline was still very prominent, as is also illustrated by Fig. 5-7. From these results, it was concluded that the integrity of noradrenaline storage by the intraneuronal granules was not an essential pre-requisite for delayed recovery.

Effect of drugs applied during delayed recovery:

The alpha-receptor blocking agent, phentolamine (0.1 µg/ml) was added extraluminally during delayed recovery in 2 experiments and Fig. 5-8 illustrates its effect in rapidly reducing the perfusion pressure to its resting level. Cocaine (1 µg/ml) applied extraluminally at any stage of delayed recovery, had the opposite effect in that it caused a marked rise in perfusion pressure (Fig. 5-9). This latter observation was repeated many times and was also seen during delayed recovery in monoamine oxidase-inhibited arteries removed from reserpine-pretreated rabbits (Fig. 5-7). Cocaine consistently failed to elicit a response, however, when the perfusion pressure was at its resting level, both in untreated and monoamine-oxidase-inhibited arteries. Cocaine has been shown previously to markedly enhance the constrictor responses

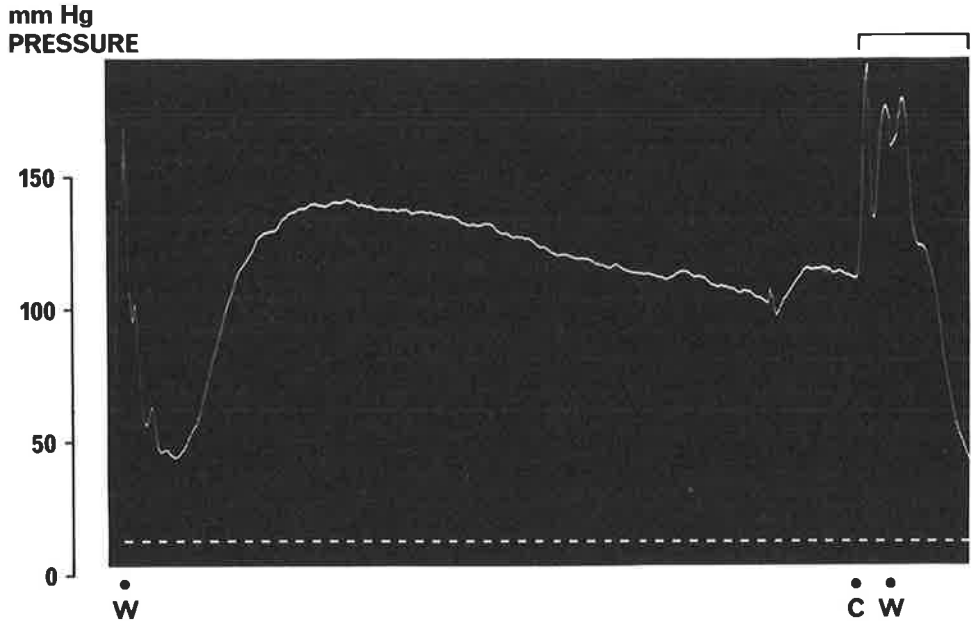


Fig. 5-7 Delayed recovery from the response to prolonged (30 minute) exposure to extraluminal noradrenaline (0.5 µg/ml) in a nialamide-treated artery which had been removed from a reserpine-pretreated rabbit. Cocaine (C) in a concentration of 1 µg/ml, applied extraluminally during the recovery phase, caused a marked rise in perfusion pressure. W refers to standard washout of the extraluminal drugs. The base-line is represented by the horizontal interrupted white line.

Time scale: 10 minutes.

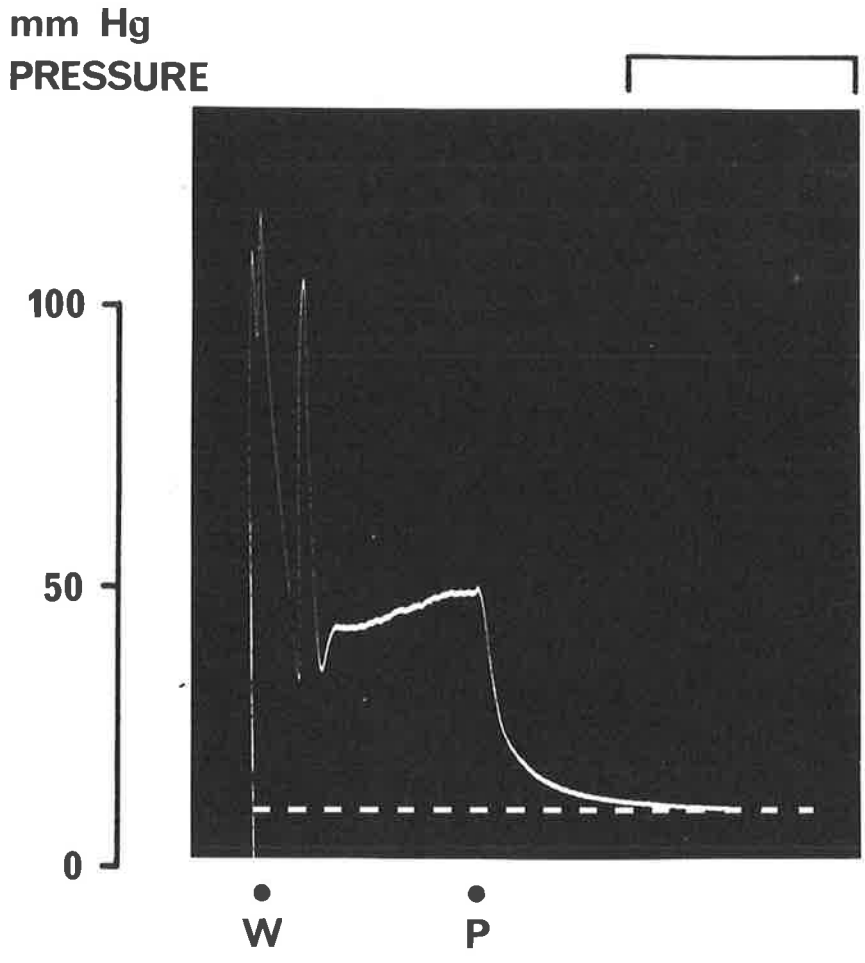


Fig. 5-8 Delayed recovery from the response to prolonged (30 minute) exposure to extraluminal noradrenaline (0.5 µg/ml) in a nialamide-treated artery. The extraluminal application of phentolamine (P), in a concentration of 0.1 µg/ml, is seen to rapidly abolish the delayed recovery phase. W refers to standard washout of the extraluminal drugs. The base-line is represented by the horizontal interrupted white line.

Time scale: 10 minutes.

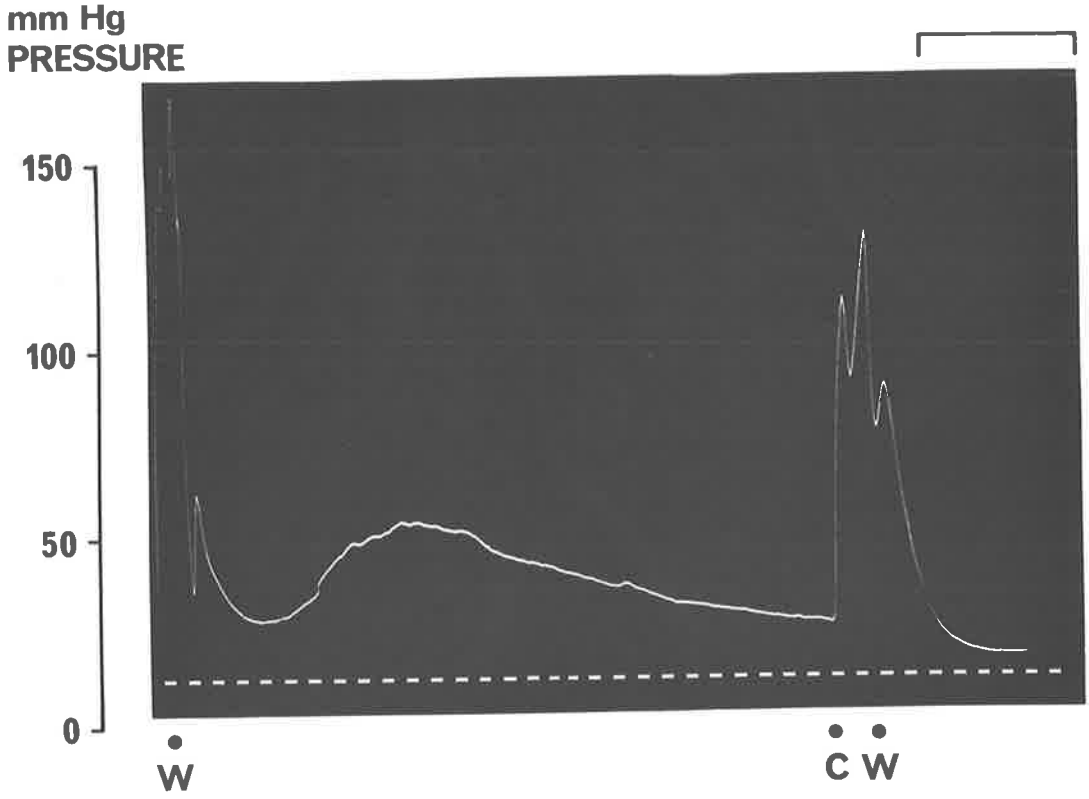


Fig. 5-9 Delayed recovery from the response to prolonged (30 minute) exposure to extraluminal noradrenaline (0.5 µg/ml) in a nialamide-treated artery. The extraluminal application of cocaine (C) in a concentration of 1 µg/ml is seen to cause a marked rise in perfusion pressure. W refers to standard washout of the extraluminal drugs. The base-line is represented by the horizontal interrupted white line.

Time scale: 10 minutes.

to exogenous or endogenously-released noradrenaline in the ear artery (de la Lande and Waterson, 1967; de la Lande, Frewin and Waterson, 1967). Hence the above changes of perfusion pressure seen with phentolamine and cocaine are consistent with the presence of extraneuronal noradrenaline in the artery wall during the phase of delayed recovery. In all cases, following washout of the cocaine applied during delayed recovery, the perfusion pressure tended to decrease below that prevailing prior to addition of the drug, but the overall effect on the time course of delayed recovery was slight. Nevertheless, the constrictor response to cocaine implied that noradrenaline was undergoing continuous uptake by the nerve terminals during the period of delayed recovery.

Intraluminal noradrenaline in the nialamide-treated artery:

A consistent feature of all experiments was the absence of delayed recovery following perfusion of the monoamine oxidase-inhibited artery with intraluminal noradrenaline (Figs. 5-1, 5-4 and 5-5; Tables 5-1 and 5-2). Cocaine also failed to elicit a constrictor response at any stage following washout of intraluminal noradrenaline. These findings implied that uptake of intraluminal noradrenaline by the sympathetic nerve terminals was very much less than that of extraluminal noradrenaline, suggesting in turn that intraluminal noradrenaline failed to achieve concentrations in the vicinity of the nerve terminals comparable with those achieved by

extraluminal noradrenaline.

One possible explanation for the failure of the intraluminal noradrenaline to achieve high concentrations in the vicinity of the medial-adventitial border of the artery wall was that it became diluted as it diffused through to the outer border of the media by the noradrenaline-free extraluminal bathing solution. This hypothesis was examined in 5 experiments in which extraluminal Krebs bicarbonate solution was replaced with liquid paraffin B.P. at 37°C throughout the period of exposure of the artery to intraluminal noradrenaline, on the basis that noradrenaline is insoluble in liquid paraffin at physiological pH. Concurrent with cessation of intraluminal noradrenaline perfusion, the extraluminal liquid paraffin was replaced by Krebs bicarbonate solution and the rate of recovery from the noradrenaline was recorded. These results are also shown in Tables 5-1 and 5-2 where it can be seen that paraffin had no significant influence on recovery time following the administration of intraluminal noradrenaline.

These findings therefore suggested that other factors besides dilution were operative in preventing the penetration of intraluminal noradrenaline to the region of the nerve terminals. Paraffin soaking, however, may be criticized on a number of grounds, not the least of which is the possibility that the extraluminal paraffin may have diffused into the artery wall to an extent that

it constituted a barrier between the nerve terminals and the media and thus prevented uptake of intraluminal noradrenaline.

Effect of monoamine oxidase inhibition on the sensitivity to noradrenaline:

The effect of nialamide on the sensitivity to intraluminal and extraluminal noradrenaline was examined in 7 untreated innervated arteries. Concentrations of intraluminal and extraluminal noradrenaline required to produce the same response before and after perfusion for 1 hour with nialamide (100 µg/ml) were estimated. The responses were those to brief (2 to 5 minute) periods of contact of noradrenaline and the artery. In addition, Professor I. S. de la Lande made his results available from a further 6 experiments in which he compared the effect of nialamide (100 µg/ml) for 1 hour on paired innervated (control) and denervated ear arteries from 6 rabbits which had undergone sympathetic denervation of one ear 5 to 20 days previously.

In the innervated arteries, following nialamide treatment, sensitivity to extraluminal noradrenaline was estimated from the magnitude of the primary or rapid phase of the response only, since the slow and gradual nature of the secondary response made its quantitation impractical. The rate of increase in perfusion pressure due to the secondary response was, however, sufficiently slow to suggest that the maximum error it would introduce would be

unlikely to correspond to more than a 2 fold change in sensitivity. This problem did not apply to extraluminal noradrenaline in the 6 denervated arteries, nor to intraluminal noradrenaline in the innervated and denervated arteries following nialamide treatment, as the secondary response did not occur in these circumstances.

The estimates of sensitivity change were complicated by the relatively long period (70 minutes, consisting of nialamide perfusion for 1 hour and washout for 10 minutes) between the pre- and post-nialamide determinations and the tendency for untreated arteries to gain spontaneously in sensitivity during this time. In order to quantitate such a non-specific effect, a control group of 7 arteries was examined and intraluminal and extraluminal noradrenaline sensitivity was determined before and after perfusion with drug-free Krebs bicarbonate solution for the same interval of time.

The changes in sensitivity following nialamide treatment in both innervated and denervated arteries and those occurring in the control group of innervated arteries following Krebs bicarbonate perfusion, are summarized in Table 5-3. The effect of nialamide was to slightly enhance the sensitivity of most innervated arteries to intraluminal noradrenaline, the mean increases being 2.0 fold and 1.1 fold in the two separate groups of arteries, of which the latter were the controls for the denervated arteries. However, the

TABLE 5-3

Gain in sensitivity to intraluminal and extraluminal noradrenaline produced by nialamide in vitro or Krebs bicarbonate solution alone, expressed as the sensitivity ratio $\frac{\text{Before treatment}}{\text{After treatment}}$ *(1).*

Route of administration of noradrenaline	TREATMENT			
	Krebs bicarbonate solution alone (2)	Nialamide in innervated arteries A	Nialamide in innervated arteries B (3)	Nialamide in denervated arteries
Intraluminal	1.3 (1.7) (1.0)	2.0 (2.6) (1.5)	1.1 (1.3) (0.9)	1.1 (1.2) (0.9)
Extraluminal	1.2 (1.4) (1.0)	2.0 (2.6) (1.6)	Not estimated	1.5 (2.0) (1.2)
Number of arteries	7	7	7	6

(1) Ratio of equipotent noradrenaline concentrations estimated at a response level of 60 mm of mercury. The values shown are the geometric means of the ratios from separate experiments and the figures in brackets refer respectively to mean + standard error and mean - standard error. Since the means are geometric, the values of (+ standard error) and (- standard error) differ.

(2) Spontaneous changes in sensitivity after perfusion with Krebs bicarbonate solution in place of nialamide.

(3) Controls for the denervated arteries.

increases were not significant compared with the spontaneous increase in sensitivity (1.3 fold) displayed by the Krebs bicarbonate-perfused control group. The 2.0 fold gain in sensitivity to extraluminal noradrenaline produced by nialamide treatment in the innervated arteries was also not significant compared with that occurring (1.2 fold) in the Krebs bicarbonate-perfused controls. However, this figure of 2.0 fold potentiation of extraluminal noradrenaline was subject to error as the determination of the "maximum response" after nialamide treatment was complicated by the difficulty of distinguishing between the plateau of the primary response and the onset of the secondary response. In addition, since this latter mean estimate included little, if any, of the secondary response, it clearly does not represent the true effect of nialamide on extraluminal noradrenaline sensitivity in the innervated artery and it is included here only insofar as it indicates the absence of any major action of nialamide on the primary phase of the noradrenaline response. Since the denervated arteries displayed a well-defined plateau of the primary response owing to the absence of the secondary response, changes in sensitivity to both intraluminal and extraluminal noradrenaline after nialamide treatment could be estimated precisely. However, the respective changes (1.1 fold) and (1.5 fold) were not significant.

Noradrenaline fluorescence histochemistry:

Table 5-4 shows the results of 9 experiments examining the possibility of intraluminal and extraluminal noradrenaline repletion of the noradrenergic storage sites in nialamide-treated ear arteries removed from 9 reserpine-pretreated rabbits. Figure 5-10 is typical of all 9 single-cannulated arteries which had been treated with nialamide (100 µg/ml) for 1 hour and then subsequently perfused only with Krebs bicarbonate solution. It will be noted that noradrenaline fluorescence at the site of the sympathetic nerve terminals in the region of the medial-adventitial border of the vessel is completely absent, indicating effective depletion of noradrenaline by reserpine. Non-perfused ear artery segments were also removed from each animal and these similarly showed absence of noradrenaline fluorescence.

Prolonged (30 minute) intraluminal application of noradrenaline (0.5 µg/ml) to a double-cannulated artery segment removed from each of the 9 animals (Fig. 5-11) also showed absence of noradrenaline fluorescence, suggesting that intraluminal noradrenaline was unable to gain access to the sympathetic nerve terminals. Extraluminally-applied noradrenaline (0.5 µg/ml), however, was associated with moderate to marked noradrenaline fluorescence (Fig. 5-12) in all 9 double-cannulated arteries, indicating repletion of the noradrenergic storage sites. These

TABLE 5-4

Noradrenaline fluorescence in nialamide-treated ear artery segments removed from 9 reserpine pretreated rabbits. Effect of prolonged (30 minute) application respectively of intraluminal and extraluminal noradrenaline (0.5 µg/ml).

Exp. No.	Krebs-perfused reserpine control (1)	Intraluminal noradrenaline (2)	Extraluminal noradrenaline (2)
1	0	0	++
2	0	0	+++
3	0	0	+++
4	0	0	+++
5	0	0	+++
6	0	0	+++
7	0	0	+++
8	0	0	++
9	0	0	+++

(1) Single-cannulated artery segments

(2) Double-cannulated artery segments

Intensity of fluorescence:

0 = Nil

\+ = Slight

++ = Moderate (Equivalent to fluorescence in an untreated normal artery)

+++ = Marked

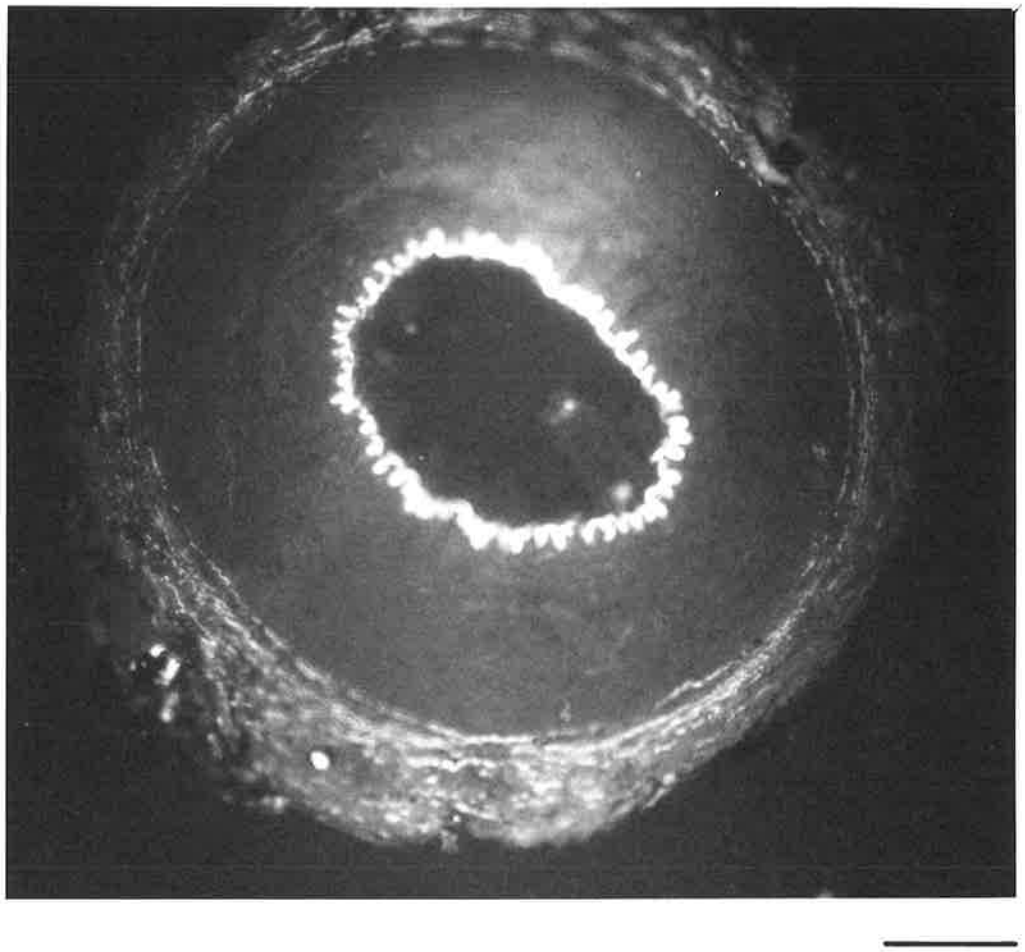


Fig. 5-10 Transverse section of a left ear artery removed from a reserpine-pretreated rabbit. The artery was single-cannulated and treated with nialamide (100 µg/ml) for 1 hour and then perfused with Krebs bicarbonate solution alone for 1 hour. Examination by the noradrenaline fluorescence technique shows absence of noradrenergic fluorescence at the medial-adventitial border of the vessel, indicating effective noradrenaline depletion by reserpine.

Section thickness: 7 microns

Formaldehyde treatment: 1 hour

Scale: 100 microns

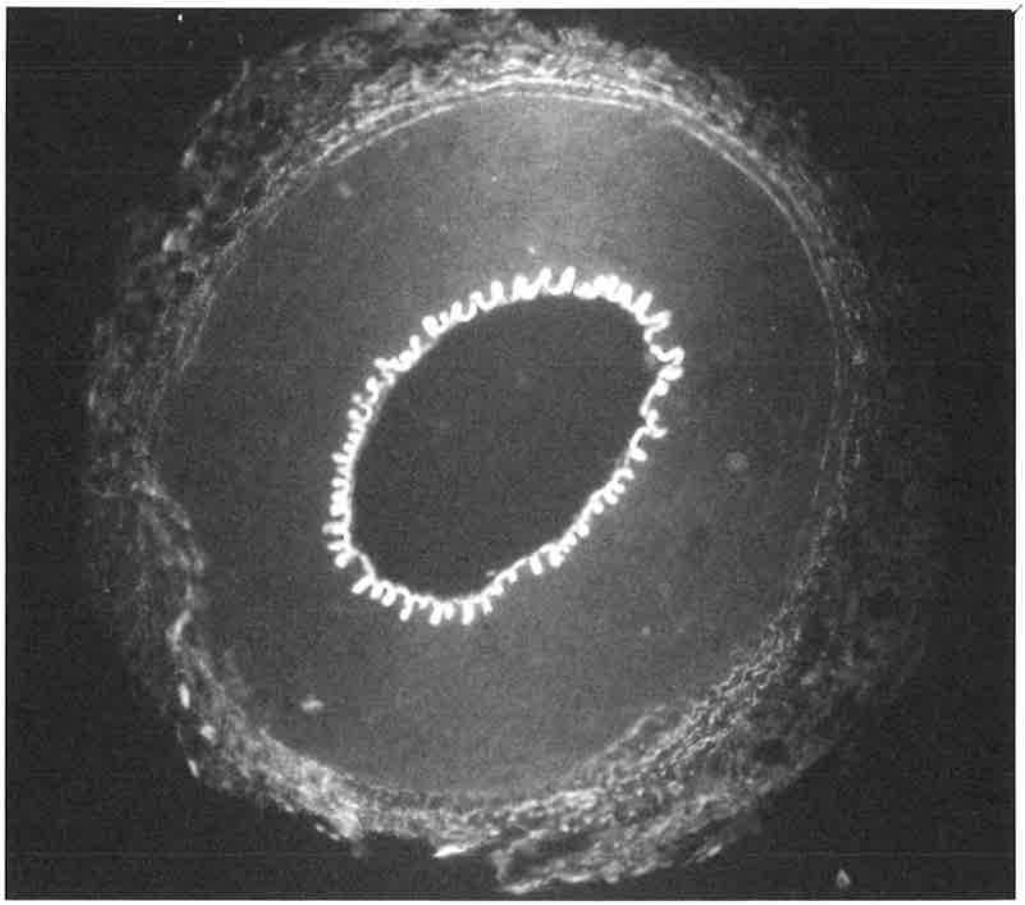


Fig. 5-11 Transverse section of a double-cannulated segment of the right ear artery removed from the same reserpine-pretreated rabbit as described in Fig. 5-10. The artery was treated with nialamide (100 µg/ml) for 1 hour and was then exposed to intraluminal noradrenaline (0.5 µg/ml) for 30 minutes. Examination by the noradrenaline fluorescence technique after washout with Krebs bicarbonate solution still shows absence of noradrenergic fluorescence at the medial-adventitial border of the vessel.

Section thickness: 7 microns

Formaldehyde treatment: 1 hour

Scale: 100 microns

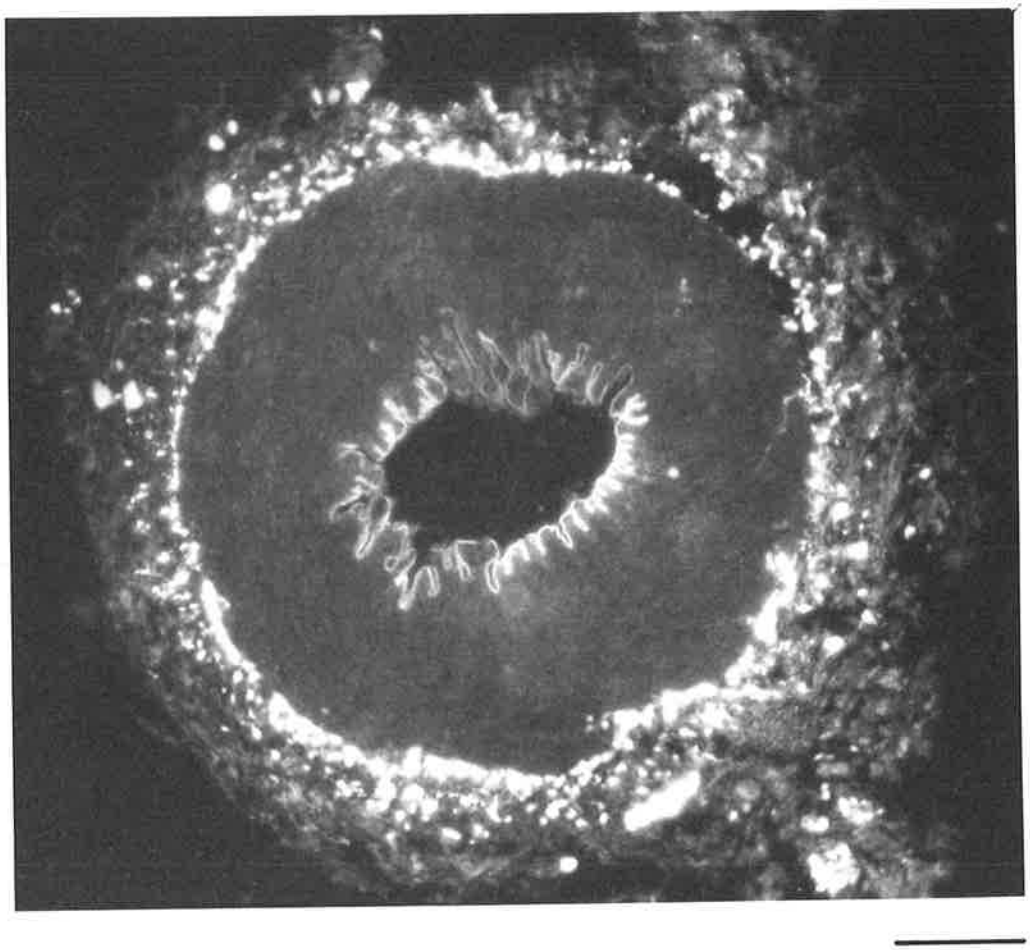


Fig. 5-12 Transverse section of a double-cannulated segment of the right ear artery removed from the same reserpine-pretreated rabbit as described in Fig. 5-10. The artery was treated with nialamide (100 µg/ml) for 1 hour and was then exposed to extra-luminal noradrenaline (0.5 µg/ml) for 30 minutes. Examination by the noradrenaline fluorescence technique after washout with Krebs bicarbonate solution shows marked noradrenergic fluorescence at the medial-adventitial border of the vessel.

Section thickness: 7 microns

Formaldehyde treatment: 1 hour

Scale: 100 microns

histochemical results are thus in accord with the findings of the physiological experiments which had tended to suggest lack of access of intraluminal noradrenaline to the sympathetic nerve terminals compared with easy access of extraluminal noradrenaline.

DISCUSSION:

The phenomena of the secondary response and delayed recovery seen with extraluminal application of noradrenaline to the monoamine oxidase-inhibited rabbit ear artery have also been described following the application of noradrenaline to the monoamine oxidase-inhibited guinea-pig atrium (Furchgott and Sanchez Garcia, 1968). The secondary response has also been noted in the cat nictitating membrane (Tsai, 1968). The former authors referred to both phenomena jointly as secondary sensitization. They attributed the phase of secondary constriction (the secondary response) to leakage of noradrenaline from its storage sites in the postganglionic sympathetic nerves and nerve terminals during exposure to exogenous noradrenaline and delayed recovery to the persistence of leakage beyond the period of exposure. They suggested that inhibition of monoamine oxidase removes one of the factors tending to maintain low cytoplasmic levels of noradrenaline in the neurones. As a result, the cytoplasmic level of noradrenaline increases to an extent which results in saturation of the intraneuronal binding structures so that free intraneuronal noradrenaline accumulates and

then diffuses out of the neurones into the surrounding medium.

This explanation of secondary sensitization in the guinea-pig atrium is supported by the observations on the rabbit ear artery obtained in the present study. Thus, cocaine and phentolamine caused constriction and dilatation, respectively, when these drugs were added after noradrenaline washout at a stage when the constrictor tone of the artery was still elevated. These effects are characteristic of the interactions of cocaine and phentolamine with exogenous noradrenaline and hence support the concept that delayed recovery is associated with the presence of free intraneuronal noradrenaline. In contrast to its effect when added after noradrenaline washout, cocaine virtually abolished both the secondary response and delayed recovery when it was present throughout the period of exposure to noradrenaline. Chronic sympathetic denervation produced a similar effect to cocaine in abolishing the secondary response and delayed recovery and in view of the evidence that cocaine inhibits uptake of noradrenaline by the sympathetic nerves in the rabbit ear artery (de la Lande and Waterson, 1967, and de la Lande, Frewin and Waterson, 1967) it must be concluded that this uptake is an essential prerequisite to delayed recovery. These findings also strongly support the probability that the structures from which noradrenaline is released are neuronal and not extraneuronal. The alternative possibility is that both cocaine

and chronic sympathetic denervation may, in some unknown way, reduce the binding of noradrenaline by structures outside of the sympathetic nerves. However, the reviews of Gillespie (1968) and Iversen (1968) offer little support for this latter hypothesis.

Cocaine and chronic sympathetic denervation also prevented the secondary response to extraluminal noradrenaline. However, these experiments were carried out at equi-effective and not equal concentrations of noradrenaline and the results may have reflected the sensitizing effects of cocaine and denervation on the response to extraluminal noradrenaline. Hence the evidence that the secondary response in the ear artery depends upon uptake of noradrenaline is less complete and rests primarily on the association of the secondary response with a subsequent delay in recovery and the evidence presented above that the latter phenomenon is mediated by the sympathetic nerves. Nevertheless, in the monoamine oxidase-inhibited nictitating membrane of the cat, Tsai (1968) indicated that the secondary response to noradrenaline was prevented by cocaine and denervation, and a subsequent study has provided strong support for the concept that the sympathetic nerves and their uptake of noradrenaline are essential prerequisites for the secondary response in this tissue (Trendelenburg, private communication, 1971).

In the present study, it was noted that reserpine pretreatment did not reduce, but instead tended to accentuate delayed

recovery in the monoamine oxidase-inhibited ear artery, implying that binding of noradrenaline by the intraneuronal storage granules is not essential for this phenomenon. There is also evidence that cytoplasmic structures other than the storage granules may bind noradrenaline in the reserpine-pretreated, monoamine oxidase-inhibited guinea-pig atrium (Furchgott and Garcia, 1968), and direct radioautographic evidence for non-vesicular binding of noradrenaline in sympathetic nerves after reserpine pretreatment and monoamine oxidase inhibition has been presented by Taxi and Droz (1969).

It is tempting to assume that the binding of noradrenaline by non-vesicular structures and its subsequent release is an important factor contributing to the delay in recovery. However, Trendelenburg favours the view that the ability of reserpine to enhance the effects of monoamine oxidase inhibition stems from the decrease in the intraneuronal binding capacity and the associated increase in the level of free cytoplasmic noradrenaline. His evidence is based on the findings that reserpine enhances the secondary response in the cat nictitating membrane (Trendelenburg, private communication, 1971) and also enhances the decline in the rate of net uptake of noradrenaline in the perfused rabbit heart which occurs after inhibition of monoamine oxidase (Graefe and Trendelenburg, 1970; Trendelenburg and Draskóczy, 1970). Of

particular interest is the evidence (Trendelenburg, private communication, 1971) that the decline in net uptake of noradrenaline by the sympathetic nerve terminal is associated with an increase in the efflux and not with a decrease in the influx of noradrenaline. The fact that the influx as well as the efflux of noradrenaline is proceeding throughout the period of delayed recovery in the rabbit ear artery is indicated in the present study by the constrictor response to cocaine and this in turn supports the concept that the re-uptake of noradrenaline after its release is a contributory factor to the prolonged nature of the delay in recovery. However, the relative contributions of this factor and that of noradrenaline released from non-vesicular binding structures cannot be decided on the basis of existing evidence.

The relationship between the findings in the ear artery and those of Kalsner and Nickerson (1968) on the rabbit aorta is less clear. These authors employed the oil immersion technique, where diffusion of extraneuronal noradrenaline from the tissue into the surrounding medium is prevented. They showed that following monoamine oxidase inhibition with iproniazid, recovery was delayed after exposure of the aorta to high concentrations of noradrenaline (1 µg/ml) or after low concentrations of noradrenaline, provided that the catechol-O-methyl transferase inhibitor, tropolone, was also present. With the higher concentration of noradrenaline, the

effect of the combination of iproniazid and tropolone in delaying recovery was not reduced when cocaine was also present, but was enhanced to a small extent. This implies that the delayed recovery caused by iproniazid in the aorta is not critically dependent upon prior uptake of noradrenaline by the sympathetic nerves as it is in the rabbit ear artery. These workers also provided other evidence to support a major role of extraneuronal enzymatic inactivation of noradrenaline in the aorta. It must be kept in mind, however, that after washout from the surrounding medium, the concentration of free extracellular noradrenaline in the tissue will decline at a relatively slow rate under conditions of oil immersion. This in turn may reduce the rate at which bound noradrenaline is released and hence reduce the relative contribution of the latter to receptor activation. Oil immersion may thus tend to exaggerate the pharmacological importance of inactivation of noradrenaline by extraneuronal enzymatic mechanisms.

Another finding in the present study was the failure of intraluminal noradrenaline to reproduce the previously described effects of extraluminal noradrenaline on the monoamine oxidase-inhibited ear artery. The time courses of the responses (including the recovery phase) to intraluminal noradrenaline were little different in control and monoamine oxidase-inhibited arteries. These time courses were similar to those of extraluminal

noradrenaline application to arteries possessing normal monoamine oxidase activity and to those of extraluminal noradrenaline application to monoamine oxidase-inhibited arteries which had been perfused simultaneously with cocaine and noradrenaline or which had been treated previously by chronic sympathetic denervation.

In view of the pharmacological evidence presented so far, suggesting that secondary sensitization depends upon uptake of noradrenaline by the nerve terminals, the failure of intraluminal noradrenaline to display secondary sensitization suggested that uptake of intraluminal noradrenaline by the nerve terminals was insufficient to produce saturation of the intraneuronal binding structures. It appeared therefore that when they were applied to the artery wall in identical concentrations, intraluminal noradrenaline failed to achieve the same concentration in the vicinity of the nerve terminals as extraluminal noradrenaline. The difference between the latter concentrations of noradrenaline was at least 10-fold, as secondary sensitization was noted to occur with extraluminal concentrations as low as 0.05 µg/ml but not with intraluminal noradrenaline in concentrations as high as 0.5 µg/ml. Subsequent to these pharmacological experiments, however, the fluorescence histochemical experiments were performed and they have provided direct histochemical confirmation that the concentration achieved by intraluminal noradrenaline in the region of the sympathomimetic

nerve terminals at the medial-adventitial border of the artery was insignificant compared with that achieved by extraluminal noradrenaline.

It is of interest though that these results in the rabbit ear artery are in contrast to those occurring in the rabbit aorta where ^{3}H-noradrenaline has been shown to penetrate from the intimal surface to the region of the adventitia (Bevan, Osher and Bevan, 1969; Bevan and Török, 1970). Different diffusion characteristics may be one of several possible explanations for the conflicting findings in these two arteries.

Several factors may contribute to the different effects seen with intraluminal and extraluminal noradrenaline in the rabbit ear artery. One possible explanation considered was that the intraluminal noradrenaline may become diluted in the vicinity of the nerve terminals by the noradrenaline-free extraluminal bathing solution. However, the experiments in which the extraluminal bathing solution was replaced with liquid paraffin for the period of exposure to intraluminal noradrenaline offered no support for this hypothesis. Another possibility is that noradrenaline undergoes loss by uptake into smooth muscle, possibly in association with metabolism by catechol-O-methyl transferase as the amine diffuses from the intima across the media towards the nerve terminals. This explanation is supported by evidence that noradrenaline in high

concentration is taken up by the smooth muscle in the media of the rabbit ear artery (Avakian and Gillespie, 1968) and more recent evidence that extraneuronal noradrenaline uptake and enzymatic metabolism have been shown to occur in the heart muscle of the rat (Lightman and Iversen, 1969). In addition, noradrenaline fluorescence histochemical evidence favouring a major role for catechol-O-methyl transferase in metabolizing noradrenaline in the media of the ear artery has been provided by the unpublished observations of de la Lande, Lazner and Mackay (personal communication, 1970).

Another possible reason for the failure of intraluminal noradrenaline to reach significant concentrations in the region of the nerve terminals may be that there is a permeability barrier to the diffusion of noradrenaline, located somewhere in the artery wall between the intima and the nerve terminals. However, it is difficult to reconcile the presence of a permeability barrier with either the ease with which cocaine, applied intraluminally, can potentiate the effects of noradrenaline when the latter is applied extraluminally, or with the tendency of a non-noradrenergic constrictor agent such as histamine to display approximately the same potency regardless of the surface of the artery to which it is applied (de la Lande, Frewin and Waterson, 1967). Further evidence against a permeability barrier is provided by the observation that the ear artery displays little difference between its sensitivities

to intraluminal and extraluminal tyramine under conditions (monoamine oxidase inhibition) where the actions of both intraluminal and extraluminal tyramine are largely indirect, that is, mediated by release of noradrenaline from the sympathetic nerve terminals. It may be significant that tyramine is not metabolized by catechol-O-methyl transferase.

Regardless of the explanation, the differences between intraluminal and extraluminal noradrenaline, described in this study, possess a major implication to the hypothesis of vascular sensitivity to noradrenaline which was proposed by de la Lande, Frewin and Waterson (1967). According to this postulate, the major factor responsible for the marked potentiation of the effects of cocaine or chronic denervation on the response to extraluminal noradrenaline, was the relative positions of the sympathetic nerve terminals and the smooth muscle in the artery wall. This possibility arises because the nerve terminals are located between the sites of application (adventitia) and action (media) of extraluminal noradrenaline, but distal to the sites of application (intima) and action (media) of intraluminal noradrenaline. An assumption of this model is that intraluminal noradrenaline can diffuse freely across the artery wall and can attain concentrations at the medial-adventitial border comparable with those achieved by extraluminal noradrenaline. Clearly this latter assumption must be modified and the additional

factor, represented by the failure of intraluminal noradrenaline to penetrate to the nerve terminals, will tend to minimize the effects of neuronal uptake on the sensitivity to intraluminal noradrenaline.

A modified hypothesis is now presented which not only takes into account this additional factor but explains the secondary sensitization phenomenon. As in the original hypothesis, the low sensitivity to extraluminal noradrenaline is attributed to uptake of the noradrenaline by the nerve terminals as it diffuses from the adventitia to the underlying smooth muscle layer. The high sensitivity to intraluminal noradrenaline is still explained by the relationship between the site of its application and the positions of the nerve terminals and the media, with the qualification that an additional mechanism (possibly uptake by smooth muscle and/or metabolism by catechol-O-methyl transferase) operates in the media to reduce the amount of noradrenaline reaching the nerve terminals.

Intraneuronal monoamine oxidase is included in this theoretical model as an important factor operating within the nerve terminals, to enable net uptake of noradrenaline to proceed for long periods of time without saturating the uptake mechanism. As shown previously in Chapter 3, the only monoamine oxidase which can be detected histochemically in the ear artery is distributed

uniformly throughout the media. However, the histochemical evidence does not exclude the presence of the enzyme in the nerve terminals since the extremely small amounts present may be below the sensitivity threshold of the histochemical method employed. Alternatively, intraneuronal monoamine oxidase may have a different substrate sensitivity to the extraneuronal enzyme and may thus be more difficult to distinguish by means of the histochemical technique. There is, however, convincing evidence (described previously) that the enzyme is in fact present within the sympathetic nerve terminals in other tissues.

Since the extraneuronal monoamine oxidase in the ear artery is distributed throughout the media, it must be assumed that the intraluminal noradrenaline is exposed to this enzyme. Hence the inability of nialamide to alter the time course of the response or the sensitivity to intraluminal noradrenaline, argues strongly against the possibility that the extraneuronal monoamine oxidase is functionally important. This argument is supported by the failure of nialamide to significantly increase the sensitivity to either intraluminal or extraluminal noradrenaline in denervated arteries, since it has also been shown in Chapter 2 that chronic sympathetic denervation has little effect on the intensity and the distribution of the monoamine oxidase in the media.

The conclusion to the drawn, therefore, from this study is

that intraneuronal but not extraneuronal monoamine oxidase is important in the control of the kinetics of the extraluminal noradrenaline response in the rabbit ear artery. It is noteworthy that the important role of intraneuronal monoamine oxidase in the control of the time course of the noradrenaline response has been derived independently by Trendelenburg and co-workers on the basis of the interactions of reserpine, pargyline, and sympathomimetic amines on the cat nictitating membrane (Trendelenburg, private communication, 1971).

SUMMARY

1. The effect of monoamine oxidase inhibition on the noradrenaline response in the rabbit ear artery was examined with particular reference to the kinetics of the response.

2. Procedures which modified the effect of enzyme inhibition, by disrupting or inhibiting uptake and binding of noradrenaline by the sympathetic nerves and nerve terminals, were applied.

3. The phenomenon of secondary sensitization, seen with prolonged exposure to extraluminal noradrenaline, depended upon monoamine oxidase inhibition in the presence of intact sympathetic nerve terminals and upon uptake of noradrenaline by these terminals.

4. Secondary sensitization did not occur following perfusion of the monoamine oxidase-inhibited artery with intraluminal noradrenaline, suggesting that intraluminal noradrenaline failed to achieve concentrations in the vicinity of the nerve terminals comparable with those achieved by extraluminal noradrenaline.

5. Direct confirmation was provided by means of noradrenaline fluorescence histochemistry which showed that intraluminal noradrenaline was unable to gain access to the sympathetic nerve terminals.

6. Several explanations for this latter finding were considered. However, the available evidence seemed to favour loss of intraluminal noradrenaline by uptake into the smooth muscle of the media, possibly in association with metabolism by catechol-O-methyl transferase.

7. This study has indicated that intraneuronal but not extraneuronal monoamine oxidase is of importance in the control of the kinetics of the extraluminal noradrenaline response.

CHAPTER 6

THE ACTION OF TRANYLCYPROMINE ON THE RABBIT EAR ARTERY

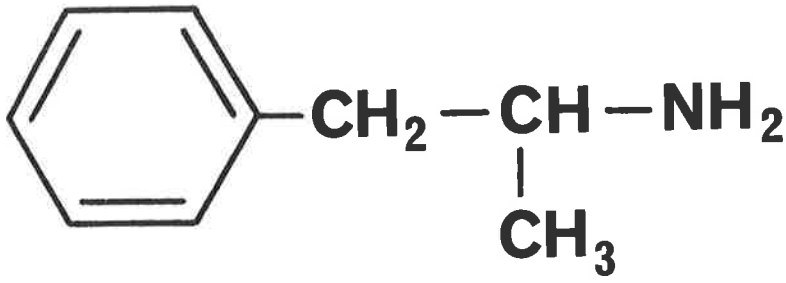
INTRODUCTION:

Tranylcypromine (SKF 385) is a non-hydrazine compound which has been shown to be a potent inhibitor of monoamine oxidase both *in vivo* (Tedeschi, Tedeschi, Cook, Mattis and Fellows, 1959; Maass and Nimmo, 1959; Sarkar, Banerjee, Ise and Zeller, 1960) and *in vitro* (Maass and Nimmo, 1959; Sarkar, Banerjee, Ise and Zeller, 1960; Zeller and Sarkar, 1962). It was found also to be considerably more potent in this respect than iproniazid (Maass and Nimmo, 1959; Green and Erickson, 1960; Zeller and Sarkar, 1962) which exemplifies the hydrazine group of irreversible monoamine oxidase inhibitors. Although iproniazid initially competes with the respective substrate for the enzyme, the action of iproniazid soon becomes non-competitive and irreversible (Pletscher, 1966). Tranylcypromine is unusual, however, because it may behave as a non-competitive inhibitor (Maass and Nimmo, 1959) or as a competitive inhibitor (Barbato and Abood, 1963), depending upon the substrate. Iproniazid requires aerobic pre-incubation with monoamine oxidase in the absence of substrate before maximum

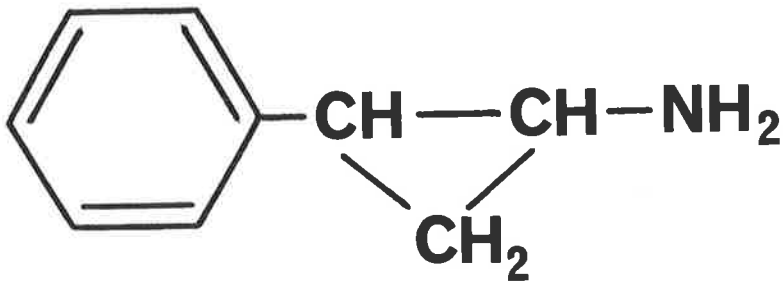
inhibitory power develops (Zeller, Barsky and Berman, 1955; Davison, 1957; Taylor, Wykes, Gladish and Martin, 1960) and this has been attributed to metabolism of the iproniazid and transformation into the active inhibitor which then reacts with the enzyme (Pletscher, 1966). It has been suggested though that tranylcypromine has a chemical affinity for the active centre of the monoamine oxidase molecule without undergoing further metabolism (Belleau and Moran, 1963).

Tranylcypromine bears a close chemical relationship to amphetamine as is illustrated by their formulae in Fig. 6-1 and was produced by cyclization of this latter drug. Amphetamine is an indirectly-acting sympathomimetic amine, releasing noradrenaline from storage sites in the sympathetic nerve terminals (Trendelenburg, Muskus, Fleming and Gomez Alonso de la Sierra, 1962a and b). There is also some experimental support for the suggestion that tranylcypromine may have a similar effect in liberating noradrenaline from sympathetic nerve terminals (Bell, 1967). Tranylcypromine, in conjunction with a large number of other drugs, including tyramine and amphetamine, has also been shown to inhibit the uptake of radioactively-labelled noradrenaline in the isolated perfused rat heart (Burgen and Iversen, 1965).

The mode of action of tranylcypromine is thus seen to have several facets. In this respect, the double-cannulated rabbit



AMPHETAMINE



TRANYLCYPROMINE

Fig. 6-1 Formulae of amphetamine and tranylcypromine.

ear artery technique seemed to offer a good experimental procedure to investigate the role which these different facets of tranylcypromine's action might play in the sensitivity of the ear artery to intraluminally- and extraluminally-applied noradrenaline. The effect of tranylcypromine on the sensitivity of the ear artery to histamine was also investigated. In contrast to exogenous noradrenaline, it has been shown (de la Lande, Frewin and Waterson, 1967) that the vasoconstrictor action of histamine on the ear artery was not associated with the presence of the noradrenergic stores and storage sites. In addition, histamine was not likely to be susceptible to monoamine oxidase in the ear artery.

The experiments described in this chapter, involving the effects of tranylcypromine on the sensitivity of the ear artery to noradrenaline, were carried out early in my investigations and prior to the studies concerning the phenomenon of secondary sensitization seen with prolonged extraluminal noradrenaline application to the nialamide-treated artery. Hence, in the present experiments, although the exposures to noradrenaline were sufficient to establish a steady-state (plateau) response, the times involved (2 to 5 minutes) were brief compared with the 30 minute exposures employed in the nialamide experiments investigating secondary sensitization.

MATERIALS AND METHODS:

Perfusion of the ear artery:

The method of isolating and perfusing the double-cannulated rabbit ear artery has been described in detail in Chapter 2. The importance of double-cannulating the artery was that comparisons could be made between vasoconstrictor responses to brief (2 to 5 minute) exposures of exogenous extraluminal and intraluminal noradrenaline. Extraluminal noradrenaline was applied to the extraluminal bathing fluid and intraluminal noradrenaline was applied either by injection into the rubber tubing just below the organ bath or by addition to the intraluminal perfusion solution (via the intraluminal reservoir).

Application of tranylcypromine to the ear artery:

Initial experiments had indicated that tranylcypromine was acting on the artery in a reversible manner, as the increased sensitivity to exogenous noradrenaline which it produced and which is described later in the Results, was eliminated by washout with drug-free Krebs bicarbonate solution for 15 minutes. Accordingly, in determining sensitivity changes produced by tranylcypromine, the drug had to be continuously present whilst these changes were being measured. The monoamine oxidase inhibitor component of tranylcypromine's action was not investigated histochemically, as the experiments described in this chapter were

performed at an early stage and prior to the establishment in the laboratory of the histochemical technique of Glenner, Burtner and Brown, Jr (1957).

Measurement of vascular sensitivity changes:

Constrictor responses to noradrenaline were recorded in duplicate at two concentration levels of the drug and dose-response curves were established as described by de la Lande, Frewin and Waterson (1967). Changes in sensitivity of the artery before and during tranylcypromine administration were established by the ratio of the noradrenaline concentrations producing constrictor responses of equal magnitude (60 mm of mercury). The sensitivity ratios expressing the change in sensitivity of the artery to histamine as a result of tranylcypromine treatment, were determined in an identical fashion. All experiments were commenced after the artery had been perfused with Krebs bicarbonate solution for 1 hour, as it had been previously shown (de la Lande and Waterson, 1968a) that the sensitivity of the artery to drugs, especially to noradrenaline, changed considerably during this time.

Sympathetic denervation:

The central ear artery of one ear was denervated in each of 5 rabbits by prior removal of the respective superior cervical ganglion by the method of de la Lande and Rand (1965). The arteries of both denervated and control ears were removed for

examination of the noradrenaline sensitivity changes produced by tranylcypromine after intervals of 14 to 21 days. The technique of denervation and the tests of its effectiveness, including noradrenaline fluorescence histochemistry, have been described in Chapter 2.

RESULTS:

Preliminary experiments with intraluminal and extraluminal noradrenaline in the ear artery revealed a tendency for extraluminal noradrenaline to be potentiated preferentially by the continuous application of tranylcypromine to the artery. Accordingly, this aspect of tranylcypromine's action was investigated in 13 experiments.

In the first 6 experiments, intraluminal and extraluminal noradrenaline dose-response curves were obtained before and during the application of tranylcypromine (100 ng/ml) and Fig. 6-2 illustrates the dose-response curves in a typical experiment. It can be seen that the shapes of the curves are sufficiently similar for the sensitivity ratios to be estimated at the one response height (60 mm of mercury). Table 6-1 shows the individual intraluminal and extraluminal noradrenaline doses eliciting responses of 60 mm of mercury before and during tranylcypromine administration and Table 6-2 illustrates the increases in noradrenaline sensitivity produced by tranylcypromine. It is apparent that the average gain

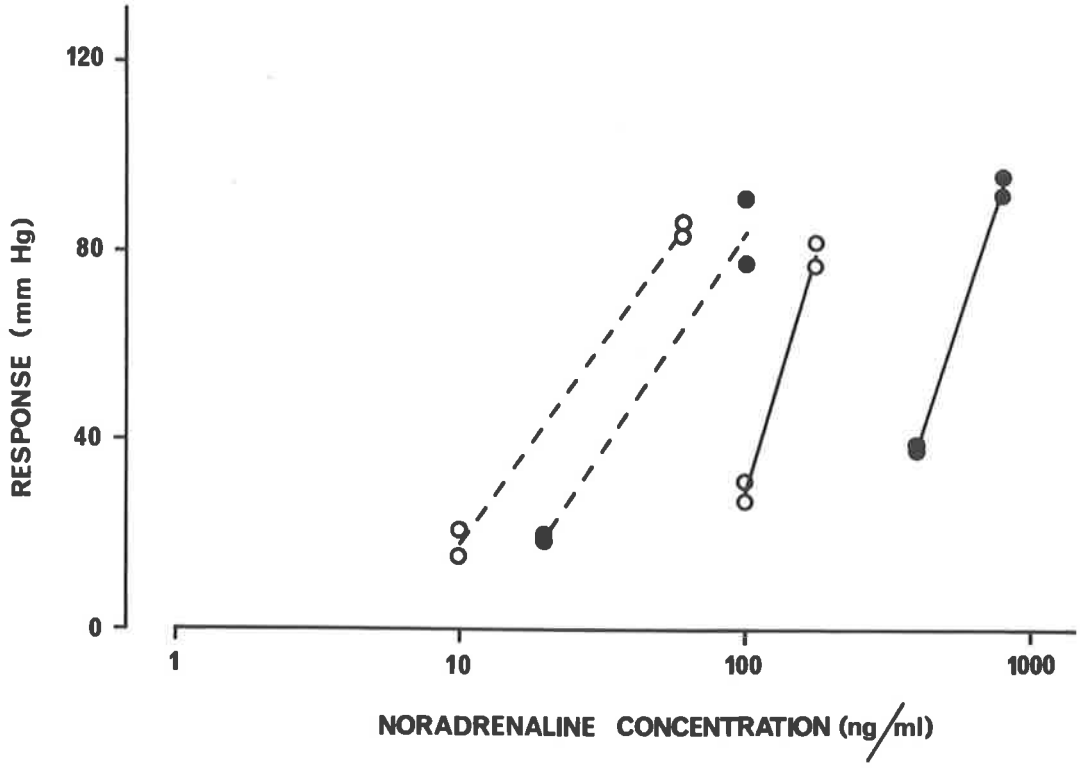


Fig. 6-2 Noradrenaline dose-response curves before (closed symbols) and during (open symbols) the continuous administration of tranylcypromine (100 ng/ml). The broken lines represent intraluminal noradrenaline and the unbroken lines extraluminal noradrenaline. Response refers to increase in perfusion pressure above the base-line (resting level).

TABLE 6-1

Sensitivity of ear arteries to noradrenaline - effect of tranylcypromine.

Doses of intraluminal (Int) and extraluminal (Ext) noradrenaline (ng/ml) producing responses of 60 mm of mercury.

Tranylcypromine (TCP) application (100 ng/ml)	Before TCP		During TCP	
Noradrenaline	Int	Ext	Int	Ext
Experiment Number 1	55.0	520	31.0	140
2	11.8	56.0	7.2	20.0
3	5.3	180	1.5	28.0
4	6.8	72.0	3.6	17.0
5	23.0	127	7.8	42.0
6	7.4	170	3.4	44.0

TABLE 6-2

Gain in sensitivity to intraluminal and extraluminal noradrenaline produced by treatment with tranylcypromine (100 ng/ml) - expressed as the sensitivity ratio, $\frac{\text{before tranylcypromine treatment}}{\text{during tranylcypromine treatment}}$, which refers to equipotent concentrations of noradrenaline producing responses of 60 mm of mercury (illustrated in Table 6-1).

Route of administration of noradrenaline	Sensitivity ratio
Intraluminal	2.2 (2.5) (2.0)
Extraluminal	3.9 (4.4) (3.4)

Each result is the geometric mean of the ratios from separate experiments and the figures in brackets refer respectively to mean + standard error and mean - standard error. Since the means are geometric, the values of (+ standard error) and (- standard error) differ.

in sensitivity to extraluminal noradrenaline (3.9 fold) was greater than that to intraluminal noradrenaline (2.2 fold).

Exactly the same methods were applied in the other 7 experiments which, however, utilized a higher concentration of tranylcypromine (500 ng/ml) and the dose-response curves of a typical experiment are illustrated in Fig. 6-3. Table 6-3 shows the individual noradrenaline doses eliciting responses of 60 mm of mercury and Table 6-4 demonstrates the respective changes in noradrenaline sensitivity. The effect of the higher tranylcypromine concentration was to enhance the differences shown at the lower concentration in that the gain in sensitivity to intraluminal noradrenaline was 4.0 fold and that to extraluminal noradrenaline was 10.7 fold with tranylcypromine (500 ng/ml).

In order to determine whether this selective potentiation of extraluminal noradrenaline was dependent upon the normal function of the sympathetic nerve terminals situated at the medial-adventitial border of the artery, two additional series of experiments were performed:

1. One ear artery in each of 5 rabbits was denervated by excision of the corresponding superior cervical ganglion 14 to 21 days prior to examination of noradrenaline sensitivity before and during the continuous application of tranylcypromine (500 ng/ml). The opposite ear artery in each case, served as a control and was

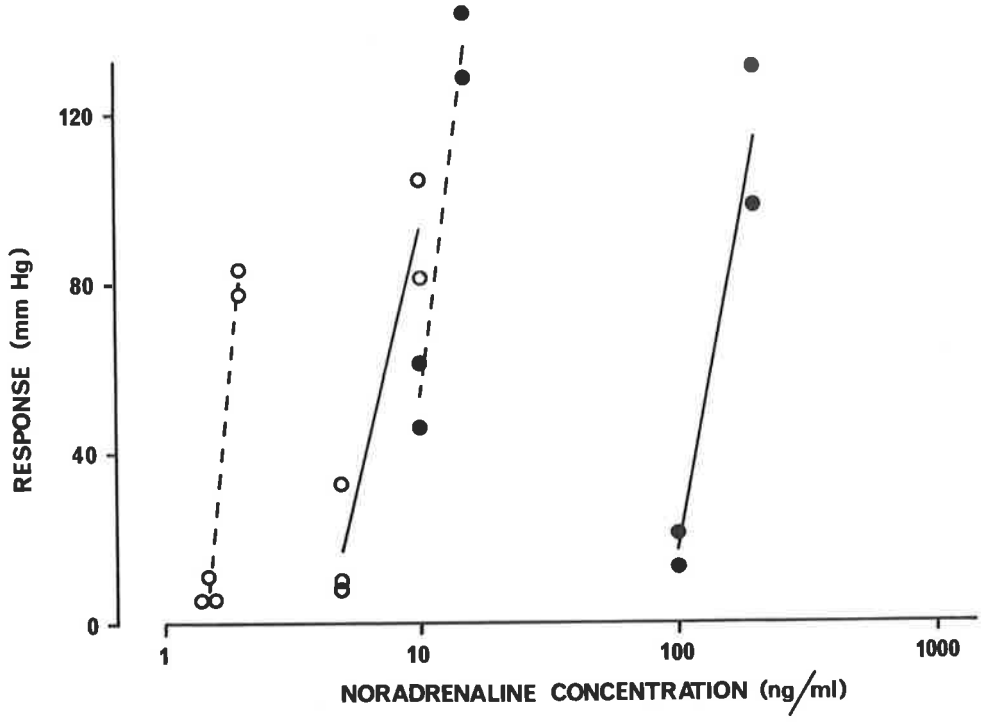


Fig. 6-3 Noradrenaline dose-response curves before (closed symbols) and during (open symbols) the continuous administration of tranylcypromine (500 ng/ml). The broken lines represent intraluminal noradrenaline and the unbroken lines extraluminal noradrenaline. Response refers to increase in perfusion pressure above the base-line (resting level).

TABLE 6-3

Sensitivity of ear arteries to noradrenaline - effect of tranylcypromine.

Doses of intraluminal (Int) and extraluminal (Ext) noradrenaline (ng/ml) producing responses of 60 mm of mercury.

Tranylcypromine (TCP) application (500 ng/ml)	Before TCP		During TCP	
Noradrenaline	Int	Ext	Int	Ext
Experiment Number 1	55.0	250	24.0	43.0
2	13.8	207	2.4	14.0
3	7.9	134	1.6	18.0
4	21.5	160	4.4	16.0
5	10.3	125	4.0	15.0
6	10.3	136	1.8	7.4
7	7.4	144	2.0	8.8

TABLE 6-4

Gain in sensitivity to intraluminal and extraluminal noradrenaline produced by treatment with tranylcypromine (500 ng/ml) - expressed as the sensitivity ratio, $\frac{\text{before tranylcypromine treatment}}{\text{during tranylcypromine treatment}}$, which refers to equipotent concentrations of noradrenaline producing responses of 60 mm of mercury (illustrated in Table 6-3).

Route of administration of noradrenaline	Sensitivity ratio
Intraluminal	4.0 (4.7) (3.5)
Extraluminal	10.7 (12.9) (9.1)

Each result is the geometric mean of the ratios from separate experiments and the figures in brackets refer respectively to mean + standard error and mean - standard error. Since the means are geometric, the values of (+ standard error) and (- standard error) differ.

treated identically. Tables 6-5 and 6-6 show that the effect of sympathetic denervation was to markedly reduce the selective potentiation of extraluminal noradrenaline which was an obvious feature in the control group of arteries.

2. The effect of tranylcypromine (500 ng/ml) on histamine-induced constriction was examined in 6 arteries. The response to histamine applied intraluminally or extraluminally, resembled that to noradrenaline in that the onset was prompt, with a rapid rise to a maximum well-sustained level within 1 to 4 minutes. The dose-response curves obtained in a typical experiment are shown in Fig. 6-4 and the equipotent concentrations of intraluminal and extraluminal histamine before and in the presence of tranyl-cypromine (500 ng/ml) and the sensitivity changes produced are illustrated respectively in Tables 6-7 and 6-8.

DISCUSSION:

The effect of tranylcypromine administration was to consistently increase the sensitivity of the rabbit ear artery to extraluminal noradrenaline to a greater extent than that to intra-luminal noradrenaline. In this respect, it closely resembled the action of cocaine on the artery. Cocaine has been shown to block the uptake of extraluminally-applied noradrenaline into the sympathetic nerve terminals situated at the medial-adventitial border of the ear artery (de la Lande and Waterson, 1967;

TABLE 6-5

Sensitivity of paired ear arteries to noradrenaline - effect of sympathetic denervation and tranylcypromine (500 ng/ml)

Doses of intraluminal (Int) and extraluminal (Ext) noradrenaline (ng/ml) producing responses of 60 mm of mercury.

	CONTROL ARTERY				DENERVATED ARTERY			
	Untreated		Tranylcypromine treatment		Untreated		Tranylcypromine treatment	
Route of administration of noradrenaline	Int	Ext	Int	Ext	Int	Ext	Int	Ext
Experiment Number 1	20.7	82.0	7.5	5.1	13.6	6.8	10.0	4.6
2	15.8	300	9.1	49.0	4.8	5.8	2.0	2.6
3	7.6	84	4.4	14.4	6.2	7.3	4.9	4.5
4	23.2	380	7.6	29.0	5.8	6.5	3.0	3.5
5	42.2	250	23.0	41.0	6.9	12.2	4.0	5.8

In this group of experiments, intraluminal (Int) noradrenaline was administered by injection.

TABLE 6-6

Effect of sympathetic denervation on the gain in sensitivity to intraluminal and extraluminal noradrenaline produced by treatment with tranylcypromine (500 ng/ml) - expressed as the sensitivity ratio, $\frac{\text{before tranylcypromine treatment}}{\text{during tranylcypromine treatment}}$, which refers to equipotent concentrations of noradrenaline producing responses of 60 mm of mercury (illustrated in Table 6-5).

Route of administration of noradrenaline	Control arteries	Denervated arteries
Intraluminal	2.2 (2.4) (1.9)	1.7 (1.9) (1.5)
Extraluminal	8.6 (10.6) (6.9)	1.8 (2.0) (1.7)

Each result is the geometric mean of the ratios from separate experiments and the figures in brackets refer respectively to mean + standard error and mean - standard error. Since the means are geometric, the values of (+ standard error) and (- standard error) differ.

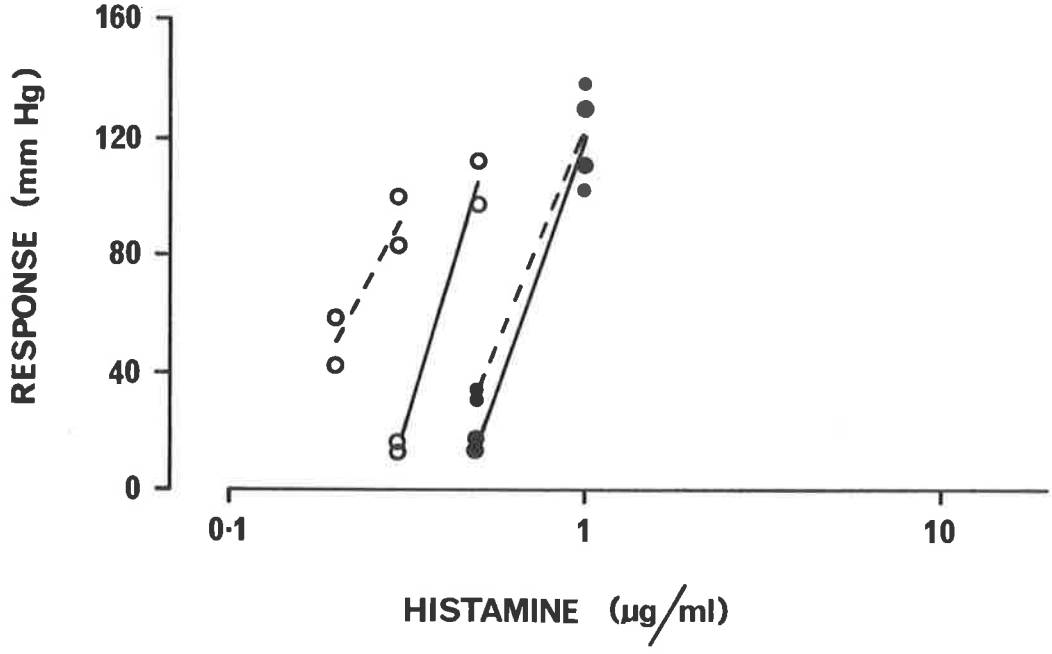


Fig. 6-4 Histamine dose-response curves before (closed symbols) and during (open symbols) the continuous administration of tranylcypromine (500 ng/ml). The broken lines represent intraluminal histamine and the unbroken lines extraluminal histamine. Response refers to increase in perfusion pressure above the base-line (resting level).

TABLE 6-7

Sensitivity of ear arteries to histamine - effect of tranylcypromine.

Doses of intraluminal (Int) and extraluminal (Ext) histamine (ng/ml) producing responses of 60 mm of mercury.

Tranylcypromine (TCP) application (500 ng/ml)	Before TCP		During TCP	
Histamine	Int	Ext	Int	Ext
Experiment Number 1	250	270	130	140
2	160	150	98	80
3	620	670	220	390
4	320	330	210	210
5	220	440	130	260
6	410	590	130	180

TABLE 6-8

Gain in sensitivity to intraluminal and extraluminal histamine produced by treatment with tranylcypromine (500 ng/ml) - expressed as the sensitivity ratio, $\frac{\text{before tranylcypromine treatment}}{\text{during tranylcypromine treatment}}$, which refers to equipotent concentrations of histamine producing responses of 60 mm of mercury (illustrated in Table 6-7).

Route of administration of histamine	Sensitivity ratio
Intraluminal	2.0 (2.3) (1.8)
Extraluminal	1.9 (2.2) (1.7)

Each result is the geometric mean of the ratios from separate experiments and the figures in brackets refer respectively to mean + standard error and mean - standard error. Since the means are geometric, the values of (+ standard error) and (- standard error) differ.

de la Lande, Frewin and Waterson, 1967), with consequent marked potentiation of the constrictor response to extraluminal noradrenaline.

The inability of tranylcypromine to selectively potentiate the extraluminal constrictor potency of the non-noradrenergic stimulant, histamine, also tended to provide confirmatory evidence for the role of the sympathetic nerve terminals and the uptake mechanism in the effect of tranylcypromine on extraluminal noradrenaline sensitivity. Further evidence supporting tranylcypromine's action in inhibiting neuronal uptake of noradrenaline has been provided by Burgen and Iversen (1965) who showed that tranylcypromine inhibited the uptake of radioactively-labelled noradrenaline in the isolated perfused rat heart.

The effect of chronic sympathetic denervation was to enhance the sensitivity of the artery to both intraluminal and extraluminal noradrenaline, with a correspondingly greater effect on the latter form of noradrenaline application, as has been described previously (de la Lande, Frewin and Waterson, 1967). Subsequent application of tranylcypromine, however, did not produce the selective increase in sensitivity to extraluminal noradrenaline which was seen in the control arteries. This absence of significant extraluminal noradrenaline potentiation can thus be attributed to the destruction of the sympathetic nerve terminals and the consequent

inability of tranylcypromine to exert its effect on noradrenaline uptake.

There is considerable evidence that tranylcypromine is a potent inhibitor of monoamine oxidase (Tedeschi, Tedeschi, Cook, Mattis and Fellows, 1959; Maass and Nimmo, 1959; Sarkar, Banerjee, Ise and Zeller, 1960; Zeller and Sarkar, 1962). It is evident, though, from the nialamide experiments described in Chapter 5, that monoamine oxidase inhibition of the ear artery caused only small and insignificant potentiation of responses to brief exposures of both intraluminal and extraluminal noradrenaline. However, apart from its cocaine-like effect on extraluminal noradrenaline sensitivity, tranylcypromine also produced 2.2 to 4.0 fold potentiation of intraluminal noradrenaline which was greater than the 1.1 to 2.0 fold potentiation of intraluminal noradrenaline produced by nialamide. As with nialamide, part of tranylcypromine's potentiating effect could be explained in terms of a spontaneous increase in sensitivity throughout the course of each experiment. However, part of the intraluminal (and also extraluminal) potentiation could possibly be due to an extraneuronal cocaine-like action as cocaine has been shown to potentiate intraluminal noradrenaline to a small degree compared with its much greater selective potentiation of extraluminal noradrenaline due to inhibition of neuronal uptake (de la Lande and Waterson, 1967; de la Lande,

Frewin and Waterson, 1967; de la Lande, Hodge, Lazner, Jellett and Waterson, 1970).

A point of interest arising from these tranylcypromine experiments was that the secondary response which was often noted with responses to brief exposures of extraluminal noradrenaline in the nialamide-treated artery, did not occur with brief extraluminal noradrenaline doses during tranylcypromine administration. This absence of secondary sensitization with tranylcypromine application is probably due to the action of tranylcypromine in blocking noradrenaline uptake into the sympathetic nerve terminals. The data in Chapter 5 have shown clearly that the noradrenaline uptake mechanism is one of the essential criteria for this phenomenon to occur.

SUMMARY

1. Tranylcypromine consistently enhanced the sensitivity of the rabbit ear artery to extraluminal noradrenaline to a greater extent than that to intraluminal noradrenaline.

2. In this respect, the action of tranylcypromine closely resembled that of cocaine in blocking uptake of noradrenaline into the sympathetic nerve terminals.

3. The inability of tranylcypromine to similarly potentiate the extraluminal constrictor potency of the non-noradrenergic stimulant, histamine, also tended to support the role of the sympathetic nerve terminals and the uptake mechanism in the effect of tranylcypromine on extraluminal noradrenaline sensitivity.

4. The selective increase in sensitivity to extraluminal noradrenaline, seen in control arteries to which tranylcypromine had been applied, did not occur in arteries which had been denervated, thus confirming the role of the sympathetic nerve terminals.

5. Tranylcypromine also produced mild potentiation of intraluminal noradrenaline. A possible explanation for this, apart from a spontaneous increase in sensitivity of the artery, may be an extraneuronal cocaine-like effect of tranylcypromine.

C H A P T E R 7

MODIFICATION OF THE VASOCONSTRICTOR ACTION OF SEVERAL SYMPATHOMIMETIC AGENTS BY BRETYLIUM TOSYLATE AND TRANYLCYPROMINE IN MAN.

INTRODUCTION:

Bretylium tosylate is an hypotensive agent whose mechanism of action is not fully understood. Its principal effect is blockade of the sympathetic noradrenergic neurones, an action considered to be due to the prevention of release of noradrenaline from the terminals of the postganglionic fibres (Exley, 1960; Laurence, 1962). Green (1962) reported that the drug had an exceptionally high affinity for sympathetic ganglia and postganglionic nerve trunks and McCoubrey (1962) found that it possessed antimonoamine oxidase activity, although this facet of its action was weak.

The vasoconstrictor actions of the sympathomimetic amines, tyramine, methylamphetamine and ephedrine, on human blood vessels have been shown to be entirely dependent on the presence of the sympathetic nerve endings (Parks, Sandison, Skinner and Whelan, 1961; Frewin and Whelan, 1968a and b). This study investigated the potentiation by bretylium of the vasoconstrictor action of these sympathomimetic amines on hand blood vessels in man and this effect

was compared with the potentiating action on these drugs of tranylcypromine which is known to have a strong monoamine oxidase inhibiting effect (Tedeschi, Tedeschi, Cook, Mattis and Fellows, 1959; Maass and Nimmo, 1959; Sarkar, Banerjee, Ise and Zeller, 1960; Zeller and Sarkar, 1962).

METHODS:

The subjects for these experiments were normal volunteer medical students. Hand blood flow was measured by venous occlusion plethysmography using water-filled plethysmographs as described in Chapter 2. The constrictor responses of the hand blood vessels to 5 minute intra-arterial infusions of tyramine (50 or 75 µg/min), methylamphetamine (10 or 20 µg/min) and ephedrine (25 or 50 µg/min) respectively, were compared in 5 experiments with each amine 15 to 20 minutes before and 30 to 50 minutes after the administration of bretylium tosylate (4 mg/min for 5 minutes) and of tranylcypromine (50 µg/min for 5 minutes). The dose of the sympathomimetic agent chosen for each subject was that expected to produce a fall in hand blood flow within the range of 20 to 50%.

Bretylium tosylate caused an initial constriction of the hand vessels in most of the experiments. This persisted for about 5 minutes after the infusion ceased. The hand blood flow then gradually rose within the next 10 minutes to or slightly above the previous resting level (Cooper, Fewings, Hodge and Whelan, 1963).

To test for the sympathetic blockade of the hand blood vessels caused by the intra-arterial infusion of bretylium, ice was applied to the neck of the subject and the resulting vasoconstriction in both hands was recorded. This procedure is a sympathetic stimulus and usually produces intense vasoconstriction in both hands (Cooper, Fewings, Hodge and Whelan, 1963). When the effects of bretylium were fully developed, the hand vessels on the treated side no longer constricted when ice was applied to the neck, while on the untreated side, vasoconstriction of the same magnitude as before was seen. Approximately 30 minutes was required in most experiments before blockade of the hand blood vessels was fully effective and the second infusion of each sympathomimetic amine was given 35 to 45 minutes after bretylium administration.

Tranylcypromine caused an initial constriction of the hand blood vessels which persisted throughout the infusion period. The blood flow returned to about the previous resting level approximately 15 minutes after the infusion ceased. A second infusion of each sympathomimetic amine was given 30 to 50 minutes after the tranylcypromine infusion, corresponding in time with those following bretylium. The effect of tranylcypromine on hand blood vessel sensitivity was also determined in 5 experiments in which noradrenaline (50 ng/min for 5 minutes) was given 10 to 15 minutes before and at 10 minute intervals after tranylcypromine

(50 µg/min for 5 minutes). The second and subsequent infusions of noradrenaline were given at a time when the blood flow had returned to the previous resting level. To conform with the time sequence which was observed in the experiments with bretylium, the percentage fall in flow caused by the noradrenaline infusion given 30 to 40 minutes after tranylcypromine was the one used in the calculations.

RESULTS:

Effect of bretylium on sympathomimetic amine responses:

The response of the hand blood flow to tyramine (75 µg/min for 5 minutes) before and then after bretylium tosylate (4 mg/min for 5 minutes) in one subject is shown in Fig. 7-1, both drugs being given by infusion into the brachial artery. The degree and duration of the constrictor response to tyramine was markedly enhanced after bretylium administration. Similar results were obtained in each of four other subjects and Fig. 7-2A shows the reduction in hand blood flow produced by tyramine in all 5 subjects expressed as percentage fall from the resting level of flow. The symbols to the left of the figure represent the values for percentage fall in flow caused by tyramine before treatment of the hand blood vessels with bretylium and those on the right of the figure represent the values after treatment. The enhancement of the vasoconstrictor action of tyramine averaged 41.4% following bretylium administration, an increase which was statistically significant

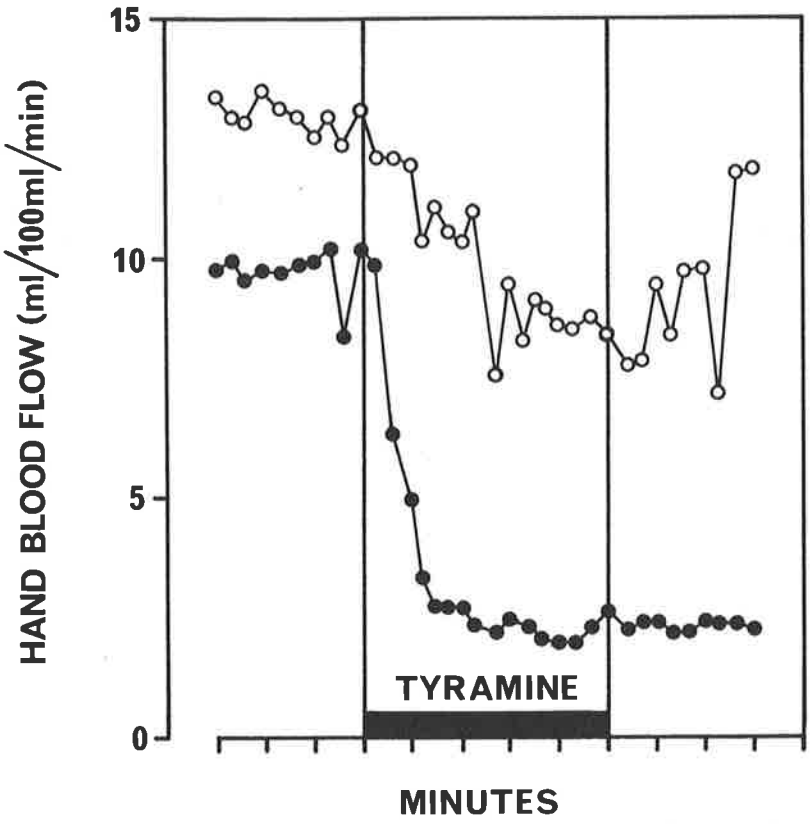


Fig. 7-1 Constrictor response of the hand blood vessels to a 5 minute intra-arterial infusion of tyramine (75 µg/min; black rectangle) 19 minutes before (○) and 40 minutes after (●) intra-arterial administration of bretylium tosylate (4 mg/min for 5 minutes).

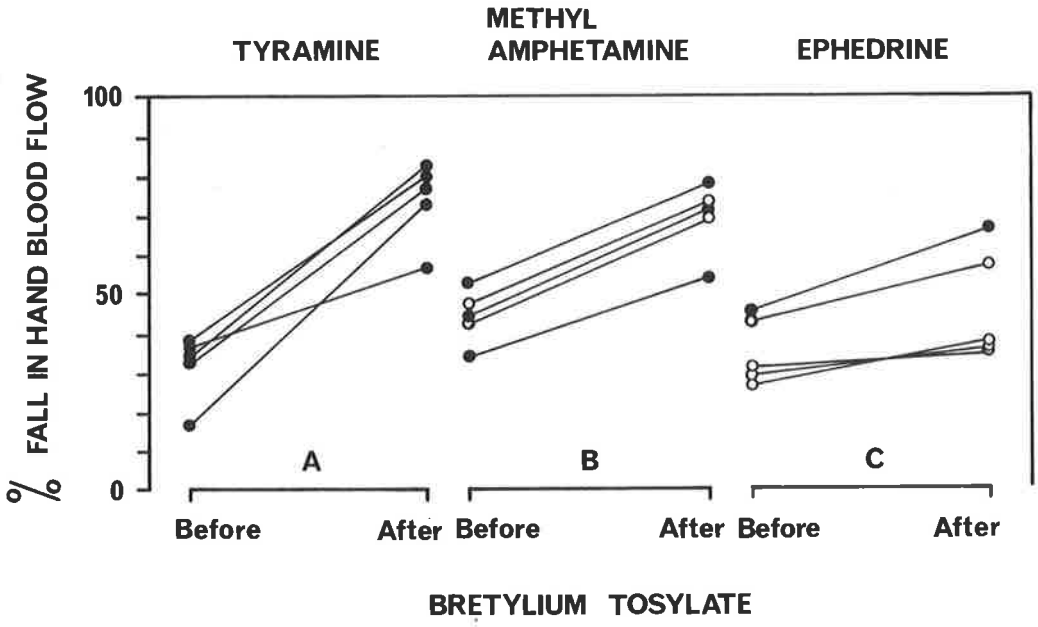


Fig. 7-2 Percentage fall in hand blood flow in response to tyramine (● = 75 µg/min), methylamphetamine (O = 10 µg/min; ● = 20 µg/min) and ephedrine (O = 25 µg/min; ● = 50 µg/min) before and after bretylium tosylate (4 mg/min for 5 minutes), shown by A, B and C, respectively. Five experiments, each on a different subject, were carried out with each sympathomimetic amine, the infusions being given 15 to 20 minutes before and 35 to 45 minutes after the administration of bretylium.

($0.0005<P<0.0025$).

Methylamphetamine was applied to each of 5 subjects before and after bretylium in a further 5 experiments and the results are shown in Fig. 7-2B. The doses of methylamphetamine employed were 20 µg/min on three occasions and 10 µg/min on two occasions. After treatment with bretylium, the enhancement of the constrictor response of the hand blood vessels to methylamphetamine was not as well marked as with tyramine, the mean value being 24.6%. The difference, however, was still highly significant ($P<0.0005$). Fig. 7-2C shows the results obtained from 5 experiments on 5 subjects in which ephedrine was given at infusion rates of 25 µg/min (on four occasions) and 50 µg/min (on one occasion). A significant enhancement of the constrictor action of ephedrine was seen after bretylium treatment, averaging 12.2% ($0.0025<P<0.005$) which, however, was smaller than that seen in the case of the other two sympathomimetic amines.

Effect of tranylcypromine on sympathomimetic amine responses:

The effects of the monoamine oxidase inhibitor, tranylcypromine (50 µg/min for 5 minutes), on the percentage falls in hand blood flow produced by 5 minute infusions of tyramine, methylamphetamine and ephedrine respectively, were also determined. An example of the response of the hand blood flow to tyramine (50 µg/min) before and after tranylcypromine administration in one

subject is illustrated in Fig. 7-3 and Fig. 7-4A shows the results from 5 experiments on 5 subjects with tyramine. In the presence of tranylcypromine, the enhancement of the constrictor response of the hand blood vessels to tyramine averaged 27.5%, this being a significant increase ($0.0005<P<0.0025$). Fig. 7-4B shows the results from 5 experiments on 5 subjects with methylamphetamine (10 µg/min) and Fig. 7-4C those from 5 experiments with ephedrine (25 µg/min). Tranylcypromine significantly enhanced the constrictor response to methylamphetamine by an average of 13.0% ($0.0125<P<0.025$), while the constrictor response to ephedrine was significantly increased by an average of 5.9% ($0.0025<P<0.005$). The means of the increases in percentage fall in hand blood flow caused by tyramine, methylamphetamine and ephedrine in the presence of bretylium and tranylcypromine are shown in Fig. 7-5. The trend in the enhancement of the constrictor responses to the sympathomimetic amines caused by both the hypotensive agent and the monoamine oxidase inhibitor are noted to be similar.

Effect of tranylcypromine on the response to noradrenaline:

In each of 5 experiments in which intra-arterial noradrenaline (50 ng/min for 5 minutes) was given before and after the administration of tranylcypromine (50 µg/min for 5 minutes), the effect of the tranylcypromine infusion (Fig 7-6) was to enhance the noradrenaline response. The average increase in response was 16.3%

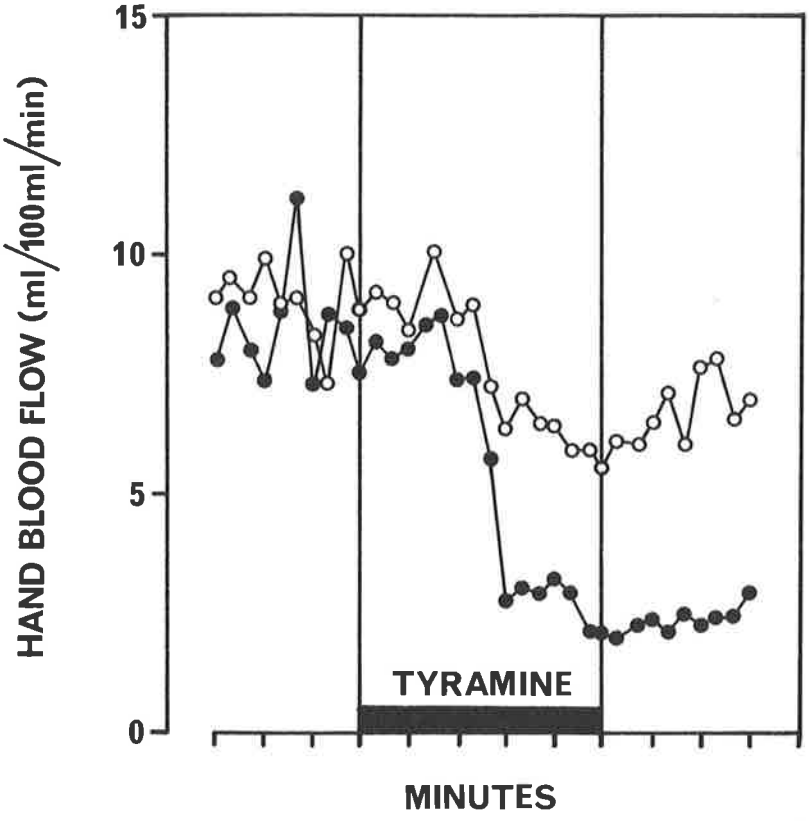


Fig. 7-3 Constrictor response of the hand blood vessels to a 5 minute intra-arterial infusion of tyramine (50 µg/min; black rectangle) 15 minutes before (○) and 45 minutes after (●) intra-arterial administration of tranylcypromine (50 µg/min for 5 minutes).

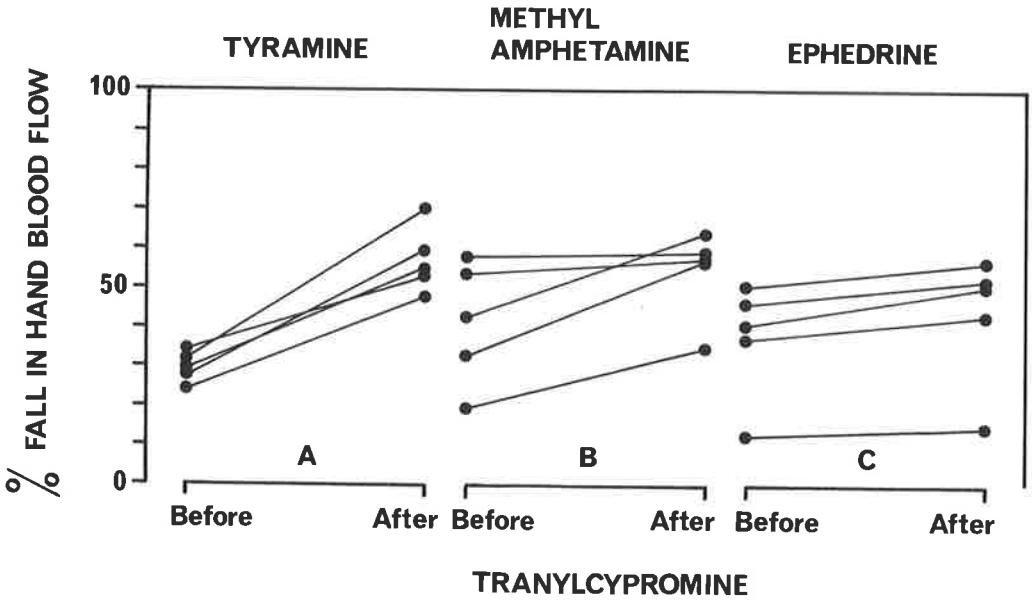


Fig. 7-4 Percentage fall in hand blood flow in response to tyramine (50 µg/min), methylamphetamine (10 µg/min) and ephedrine (25 µg/min) before and after tranylcypromine (50 µg/min) for 5 minutes, shown by A, B and C, respectively. Five experiments, each on a different subject, were carried out with each sympathomimetic amine, the infusions being given 15 to 20 minutes before and 30 to 50 minutes after the administration of tranylcypromine.

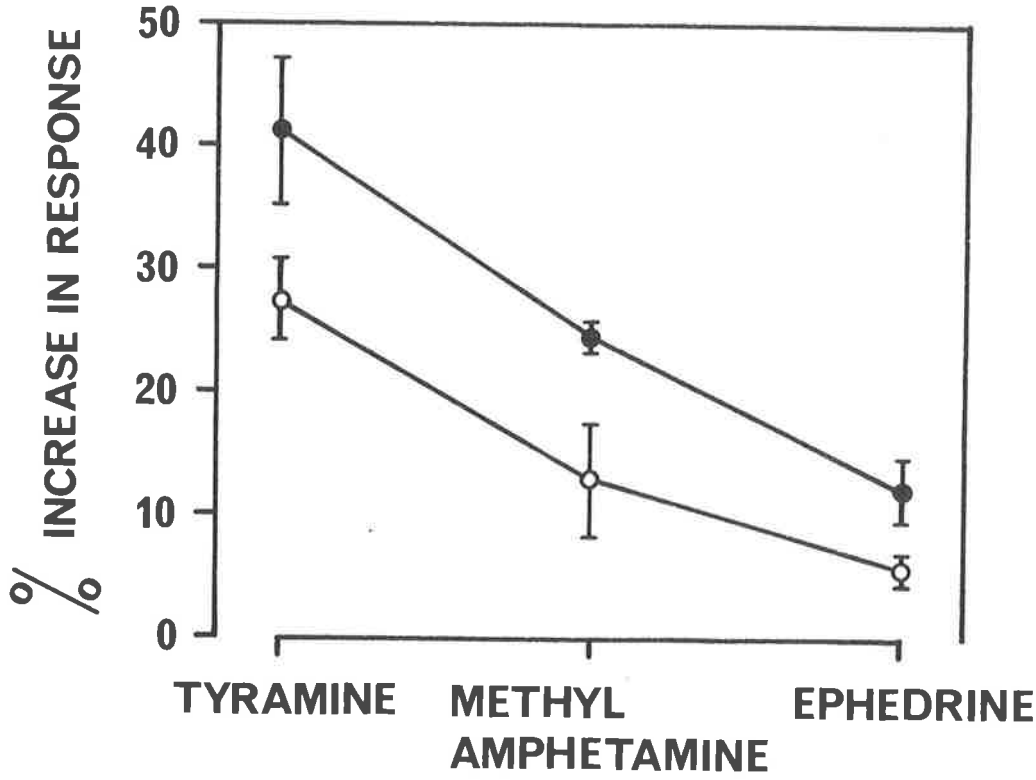


Fig. 7-5 Means of the data in Figs. 7-2 and 7-4, expressing the percentage increase in response of the hand blood vessels to tyramine, methylamphetamine and ephedrine, respectively, after bretylium (●) and after tranylcypromine (○). The vertical line through each symbol represents ± standard error of the mean.

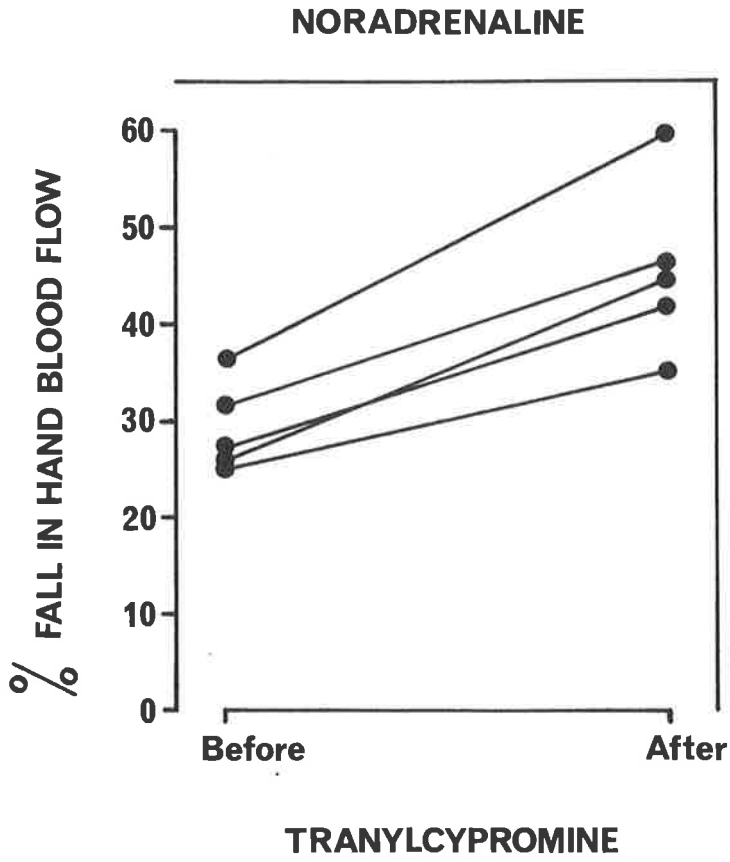


Fig. 7-6 Percentage fall in hand blood flow in each of 5 subjects in response to noradrenaline (50 ng/min for 5 minutes) before and after tranylcypromine (50 µg/min for 5 minutes).

which was statistically significant ($0.0005<P<0.0025$).

Control experiments:

Three experiments were performed in which 6 infusions of the same dose of tyramine (50 µg/min for 5 minutes) were given at intervals of 10 minutes. Saline (0.9% w/v sodium chloride solution) was continuously infused between the periods of drug administration. The constrictor responses (percentage fall in hand blood flow) were very reproducible and the mean ± standard error of the mean in each of the 3 experiments was 32.6 ± 1.4%, 33.6 ± 1.1% and 25.4 ± 1.2% respectively.

In 4 experiments, the vehicles employed as the solvents for bretylium and tranylcypromine were diluted with saline in the same manner as for the drug solutions and infused for 5 minutes at 2 ml/min. These had no effect on the blood flow through the hand, on the reflex constrictor response to ice applied to the neck, nor on the magnitude of the hand blood vessel responses to tyramine, methylamphetamine or ephedrine respectively. Responses to the latter three drugs applied consecutively were constrictions of 33.0%, 52.1% and 41.1% respectively before tranylcypromine vehicle and 36.1%, 57.2% and 43.0% respectively after the tranylcypromine vehicle. In the case of the vehicle for bretylium, the corresponding values were 45.2%, 53.7% and 56.6% before and 39.2%, 55.4% and 54.4% after the vehicle.

DISCUSSION:

In this study, the striking feature was the marked enhancement of the constrictor action of tyramine and methylamphetamine and to a lesser extent of ephedrine, on the hand blood vessels in the presence of bretylium and tranylcypromine respectively. The effect of bretylium alone on these vessels was to block reflexly-induced sympathetic activity, vasoconstriction no longer being produced in the bretylium-treated hand when ice was applied to the subject's neck. This finding is in keeping with that of Burn and Rand (1960) who observed that the action of tyramine in constricting the nictitating membrane of the spinal cat was more prolonged after the administration of bretylium at a time when the response to sympathetic nerve stimulation was abolished. Huckovič (1960) found a similar effect with tyramine after bretylium administration in the perfused rabbit ear and the isolated atrium, but the response to amphetamine of the latter preparation was not potentiated.

Wilson and Long (1960) demonstrated that when bretylium was administered to hypertensive patients on amphetamine therapy for weight reduction, no hypotensive response to the drug could be obtained. This effect was attributed to antagonism of the hypotensive action of bretylium by amphetamine. In view of the results of the present investigation, it might also be related

to the potentiating action of bretylium on the peripheral vascular action of the amine. The mechanism of enhancement of tyramine's constrictor response by bretylium is not clear. It is unlikely to be related to the continuous release of noradrenaline from the nerve endings because at the time that the tyramine and the other sympathomimetic amines were given, the constrictor effect of bretylium had worn off and the flow had returned to, or slightly above, the resting level. At this time, also, it has been shown that there is no change in sensitivity of the vessels to infused noradrenaline (Cooper, Fewings, Hodge and Whelan, 1963).

The enhancement of tyramine's action may be related to the monoamine oxidase inhibiting property which bretylium is said to possess. There is recent evidence to support such a facet of the action of bretylium (Giachetti and Shore, 1967). The effect of such an action would be threefold: (a) to protect tyramine from degradation by extraneuronal monoamine oxidase in the media of the vessel as it passes from the intima to the sympathetic nerve terminals at the medial-adventitial border of the artery (as discussed in Chapter 4); (b) to protect tyramine from intra-neuronal monoamine oxidase; and (c) to protect the noradrenaline that is released by tyramine from inactivation by the enzyme within the nerve terminals. These three effects, taken together, imply that a greater amount of noradrenaline would be released

from the storage sites by tyramine in the presence of bretylium. It is noteworthy that Pettinger and Oates (1968), from their experiments with monoamine oxidase inhibition in man, suggested that the supersensitivity seen with tyramine during monoamine oxidase inhibition, was primarily due to the interaction of tyramine with the release mechanism in the sympathetic nerve terminal, producing increased release of noradrenaline to the receptors.

While reduction in breakdown as a result of monoamine oxidase inhibition might account, at least in part, for the potentiation of tyramine's action, such a mechanism would be unlikely to apply in the case of the other sympathomimetic amines, methylamphetamine and ephedrine, which are not substrates of the enzyme. In the case of these drugs the potentiation must be accounted for in other ways and, as with amphetamine (Pettinger and Oates, 1968), potentiation of their action by monoamine oxidase inhibitors may be due solely to inhibition of intraneuronal breakdown of noradrenaline, providing an enhancement of the store available for release. Rand and Trinker (1968) have presented evidence to show that monoamine oxidase inhibitors potentiate the pressor responses of indirectly acting sympathomimetic amines, not by interfering with the metabolism of endogenous noradrenaline, but by retarding the binding and/or breakdown of these amines within the liver microsomal enzyme system. In the present

experiments, however, bretylium and tranylcypromine potentiated the constrictor action of tyramine, methylamphetamine and ephedrine on the blood vessels of the hand when the drugs were given by local intra-arterial injection and this effect cannot be attributed to any action on the liver. If the potentiating effect of bretylium is due to monoamine oxidase inhibition, this must be a local action at the peripheral nerve endings or vessel wall.

It is not clear why the sympathetic nerves, after bretylium treatment, are capable of releasing noradrenaline in response to the indirectly-acting sympathomimetic amines and yet do not do so in response to reflex activation. It may be that nerve impulses and the amines release transmitter either by different release mechanisms or from separate stores within the nerve terminal. A selective blocking action of bretylium on the nervously-activated store or mechanism, coupled with a monoamine oxidase-inhibiting action on the amine activated store or mechanism, could account for the observed effects.

The potentiation of the response of the hand blood vessels to the sympathomimetic amines by tranylcypromine could, as in the case of bretylium, be attributed to its monoamine oxidase-inhibiting property. However, unlike bretylium, tranylcypromine also inhibits noradrenaline uptake into the sympathetic nerve terminals (Burgen and Iversen, 1965) and prevention of noradrenaline uptake following

its release by the indirectly-acting sympathomimetic amines could possibly contribute to the potentiating effect of tranylcypromine. Tranylcypromine was also seen to enhance the response of the hand blood vessels to noradrenaline and this effect may also be a factor in its potentiation of the effect of the three sympathomimetic amines. However, the parallelism in the pattern of enhancement of the constrictor response of the hand blood vessels to tyramine, methylamphetamine and ephedrine in the presence of both bretylium and tranylcypromine suggests that monoamine oxidase inhibition may be a common factor in the potentiating actions of the latter two drugs.

SUMMARY

1. The vasoconstrictor actions of tyramine, methylamphetamine and ephedrine on the blood vessels of the human hand were potentiated by the intra-arterial administration of the noradrenergic neurone blocking agent, bretylium tosylate.

2. One mechanism suggested for the enhancement of vasoconstriction is that bretylium possesses monoamine oxidase inhibiting activity, which, in the case of tyramine, is protective both to the sympathomimetic amine and to the intraneuronal noradrenaline which it releases. In the case of methylamphetamine and ephedrine, which are not substrates of the enzyme, protection of the intraneuronal noradrenaline alone might occur, accounting for the lesser degree of potentiation of the effect of these amines by bretylium.

3. Comparison of the influences of bretylium and the monoamine oxidase inhibitor, tranylcypromine, on the vasoconstrictor action of the sympathomimetic amines showed a similar pattern of enhancement in the presence of both of these drugs.

4. Tranylcypromine caused enhancement of the response of the hand blood vessels to noradrenaline and this action could contribute to its potentiation of the effect of the

sympathomimetic amines.

5. For a monoamine oxidase-inhibiting action of bretylium to be effective in potentiating the constrictor actions of the sympathomimetic amines on the hand blood vessels at a time when reflex sympathetic activity is blocked, it is necessary to postulate that these drugs and reflex nerve activity act either on different compartments of the noradrenaline transmitter store or by different release mechanisms.

GENERAL SUMMARY

An historical survey of the fate of amines in the mammalian body has been presented in the introduction of this thesis and the role of monoamine oxidase in particular, has been considered in detail. Monoamine oxidase is a collective term embracing a number of isoenzymes which vary in their relative proportions in different mammalian tissues and certain metabolic effects in such tissues may possibly be related to involvement of specific and separate forms of monoamine oxidase.

Recent advances in the knowledge of noradrenaline uptake mechanisms and the pharmacology of monoamine oxidase and catechol-O-methyl transferase have been incorporated into the review of the physiological action and fate of noradrenaline at the noradrenergic nerve terminal. The concept of noradrenaline storage pools has provided a logical means of interpreting some of the dynamic changes which occur within the nerve terminal, although there is much that requires further clarification.

The rabbit ear artery has been used extensively in studies on the control of vascular sensitivity and one of the main purposes of this thesis has been to further explore the role which monoamine oxidase plays in the mechanisms involved. In order to do this, it was first necessary to investigate the distribution

of the enzyme in the ear artery and this aspect was studied in some detail by histochemical methods. Extraneuronal monoamine oxidase was demonstrated mainly throughout the media and chronic sympathetic denervation did not perceptibly alter its concentration in this region.

There was a surprising inability to observe any accumulation of the enzyme at the medial-adventitial border of the vessel, as the sympathetic nerve terminals, which are known to contain monoamine oxidase, are concentrated at this site. It may be, however, that the small size of the nerve terminals precludes their detection by the sensitivity of the histochemical method employed and by the resolution of the light microscope. An alternative explanation may be that the affinity of intraneuronal monoamine oxidase for the tryptamine hydrochloride substrate in the histochemical procedure, is less than that of extraneuronal monoamine oxidase.

In order to further assess the role of monoamine oxidase in the ear artery and also the effect on vascular sensitivity of its distribution in the artery wall, the physiological effects of several sympathomimetic agents and monoamine oxidase inhibitors were studied in detail. The sympathomimetic amine, tyramine, has been shown by others to have largely an indirect action on smooth muscle and the indirect component is mediated by release of

noradrenaline from the noradrenergic storage structures in the sympathetic nerve terminal. The experiments performed previously in this laboratory have shown that the ear artery is much more sensitive to extraluminally-applied tyramine than to intraluminally-applied tyramine.

Three possible explanations for the lower sensitivity of the artery to intraluminal tyramine had been previously postulated, namely: (1) a permeability barrier located somewhere between the intima and the medial-adventitial border of the artery; (2) a major site of loss (possibly represented by monoamine oxidase in the media) located between the intima and the medial-adventitial border and (3) dilution of the intraluminally-applied tyramine in the vicinity of the medial-adventitial border by the tyramine-free solution bathing the adventitia externally. Accordingly, the effects of monoamine oxidase inhibition on the sensitivity of the ear artery to tyramine were investigated by means of the inhibitors, iproniazid and nialamide.

Monoamine oxidase inhibition was found to enhance the sensitivity of the ear artery to both intraluminal and extraluminal tyramine. The gain in sensitivity to intraluminal tyramine, however, was much greater than that to extraluminal tyramine and the difference between the intraluminal and extraluminal potencies of the drug was much less marked after monoamine oxidase

inhibition. In view of the extraneuronal monoamine oxidase present throughout the media of the artery wall, it is suggested that the high ratio of activity of extraluminal tyramine to intraluminal tyramine which normally prevails, is due to enzymatic destruction (by monoamine oxidase) of the intraluminally-applied tyramine as it diffuses from the intima to the sympathetic nerve terminals situated at the medial-adventitial border of the artery.

Experiments were also performed in an attempt to elucidate the possible role of monoamine oxidase in the response of the rabbit ear artery to noradrenaline. It was thought possible that intraneuronal monoamine oxidase may influence the response of the ear artery to intraluminal noradrenaline in a different fashion than that to extraluminal noradrenaline. Results from other centres have been conflicting, in that intraneuronal monoamine oxidase has been assigned an important role in the response of the guinea-pig atrium to noradrenaline, whereas extraneuronal catechol-O-methyl transferase and monoamine oxidase were considered to be of greater importance in the response of the rabbit aortic strip to noradrenaline.

The effect of monoamine oxidase inhibition on the noradrenaline response in the rabbit ear artery was examined with particular reference to the kinetics of the response and procedures which modified the effects of enzyme inhibition by disrupting or

inhibiting uptake and binding of noradrenaline by the sympathetic nerves and nerve terminals, were also applied. Such techniques included destruction of the nerves by chronic sympathetic denervation, inhibition of noradrenaline uptake by cocaine and depletion of the noradrenaline stores by reserpine.

The phenomenon of secondary sensitization, reported by others to occur following the application of noradrenaline to the monoamine oxidase-inhibited guinea-pig atrium, was also noted with extraluminal application of noradrenaline to the monoamine oxidase-inhibited rabbit ear artery. Secondary sensitization was manifested by a secondary response (reported also in the cat nictitating membrane) and by delayed recovery following washout of noradrenaline. Both the secondary response and delayed recovery were abolished by chronic sympathetic denervation or by cocaine applied concurrently with the extraluminal noradrenaline. Uptake of noradrenaline by the sympathetic nerve terminals thus appeared to be essential for the occurrence of secondary sensitization.

The findings also implied that noradrenaline was released continuously from the nerve endings during the phase of delayed recovery, as cocaine added at this stage, caused a further increase in tone of the artery. In addition, the alpha-receptor blocking agent, phentolamine, abolished delayed recovery by

rapidly restoring the resting tone of the artery, when applied during this phase. Reserpine pretreatment in the monoamine oxidase-inhibited artery did not reduce, but tended to enhance both delayed recovery and also the constrictor response to cocaine applied during delayed recovery. It is possible, therefore, that the situation in the monoamine oxidase-inhibited rabbit ear artery resembles that described in the monoamine oxidase-inhibited guinea-pig atrium. Thus, inhibition of intraneuronal inactivation of noradrenaline causes saturation of the intraneuronal binding sites so that "free" noradrenaline accumulates within the cytoplasm of the neurone and eventually diffuses out into the region of the receptors. This explanation would therefore account for both the secondary response and delayed recovery, the latter phenomenon being attributed to the continuous diffusion of noradrenaline from the nerve terminal after noradrenaline had been washed out from the surrounding medium.

Surprisingly, these pharmacological studies indicated clearly that intraluminally-applied noradrenaline did not cause secondary sensitization in the monoamine oxidase-inhibited ear artery. These findings were directly confirmed by fluorescence histochemical experiments which showed that in monoamine oxidase-inhibited ear arteries removed from reserpine-pretreated rabbits, the concentration achieved by intraluminal noradrenaline in the

region of the sympathetic nerve terminals at the medial-adventitial border of the artery, was insignificant compared with that achieved by extraluminal noradrenaline. Several factors may contribute to the inability of intraluminal noradrenaline to achieve a significant concentration in the nerve terminals, including: (1) dilution of intraluminal noradrenaline by the noradrenaline-free extraluminal solution; (2) uptake of intraluminal noradrenaline into the smooth muscle of the media with subsequent metabolism by catechol-O-methyl transferase and (3) a possible permeability barrier to the diffusion of intraluminal noradrenaline, located somewhere between the intima and the region of the nerve terminals. However, others in this laboratory have subsequently shown, by similar fluorescence histochemical methods, that the major role is played by catechol-O-methyl transferase which metabolizes intraluminally-applied noradrenaline in the media.

The effect of monoamine oxidase inhibition on the magnitude of intraluminal and extraluminal noradrenaline responses was also studied and this indicated that the mean increases in sensitivity were only slightly above those which occurred spontaneously. This implies that metabolism of noradrenaline by monoamine oxidase in the media exerts little effect on the pharmacologically effective concentrations of noradrenaline at the noradrenergic receptors in the media. It is likely, therefore, that the very small quantity

of intraneuronal enzyme present within the nerve terminals plays a much more important role in the kinetics of the response to extraluminal noradrenaline than does the extraneuronal monoamine oxidase in the media.

Experiments investigating the action of tranylcypromine on the rabbit ear artery revealed consistent selective enhancement of extraluminal noradrenaline sensitivity. In this respect, the action of tranylcypromine closely resembled that of cocaine in inhibiting neuronal uptake of noradrenaline. Tranylcypromine failed to potentiate the extraluminal constrictor potency of the non-noradrenergic stimulant, histamine, and this also tended to support the role of the sympathetic nerve terminals and the neuronal uptake mechanism in the action of tranylcypromine on extraluminal noradrenaline sensitivity in the ear artery. Sympathetic denervation abolished the selective increase in sensitivity to extraluminal noradrenaline seen in the control arteries to which tranylcypromine had been applied, thus confirming the role of the sympathetic nerve terminals.

The effect of monoamine oxidase inhibition was also examined in man by comparing the influences of bretylium tosylate and tranylcypromine on the vasoconstrictor actions of various indirectly-acting sympathomimetic amines. The intra-arterial administration of the noradrenergic neurone blocking agent,

bretylium tosylate, was found to potentiate the vasoconstrictor effects of tyramine, methylamphetamine and ephedrine. It is suggested that this potentiating action of bretylium is due to monoamine oxidase inhibition and in the case of tyramine, which is a good substrate for monoamine oxidase, inhibition of the enzyme would increase the effective concentration of both tyramine and the noradrenaline which it releases. Methylamphetamine and ephedrine, however, are not substrates of monoamine oxidase and only enhancement of the noradrenaline concentration alone would occur, thus accounting for the lesser potentiation of their vasoconstrictor actions by bretylium.

The principal effect of bretylium is blockade of the sympathetic noradrenergic neurones. Hence, for bretylium to be effective as a monoamine oxidase inhibitor in potentiating the vasoconstrictor actions of these sympathomimetic amines on hand blood vessels at a time when reflex sympathetic activity is blocked, it is necessary to postulate that these drugs and reflex nerve activity act either on different intraneuronal noradrenergic storage compartments or by different release mechanisms. Comparison of the intra-arterial effects of bretylium and the monoamine oxidase inhibitor, tranylcypromine, on the vasoconstrictor action of the three sympathomimetic amines, showed a similar pattern of enhancement, suggesting that monoamine oxidase

inhibition may be a common factor in their potentiating actions. Tranylcypromine, however, also increased the response of the hand blood vessels to noradrenaline and this action could also contribute to its potentiation of the effects of tyramine, methylamphetamine and ephedrine.

A P P E N D I X

APPARATUS AND TECHNIQUES

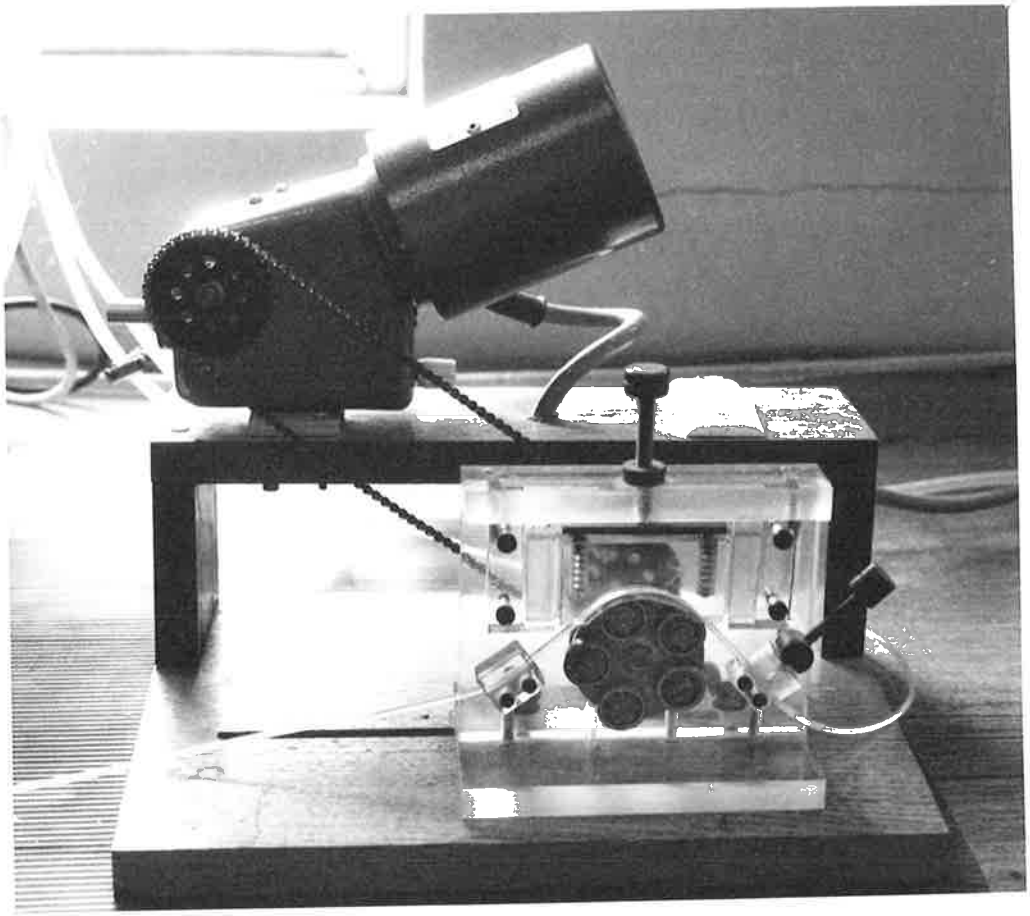
A. Perfusion experiments:

1. The proximal ear artery cannula consisted of a short length of No. 3 Sterivac polythene tubing, one end of which had been drawn out and tapered in a flame. The distal artery cannula was a U-shaped length of No. 2 Sterivac polythene tubing, one end having been tapered in a flame.

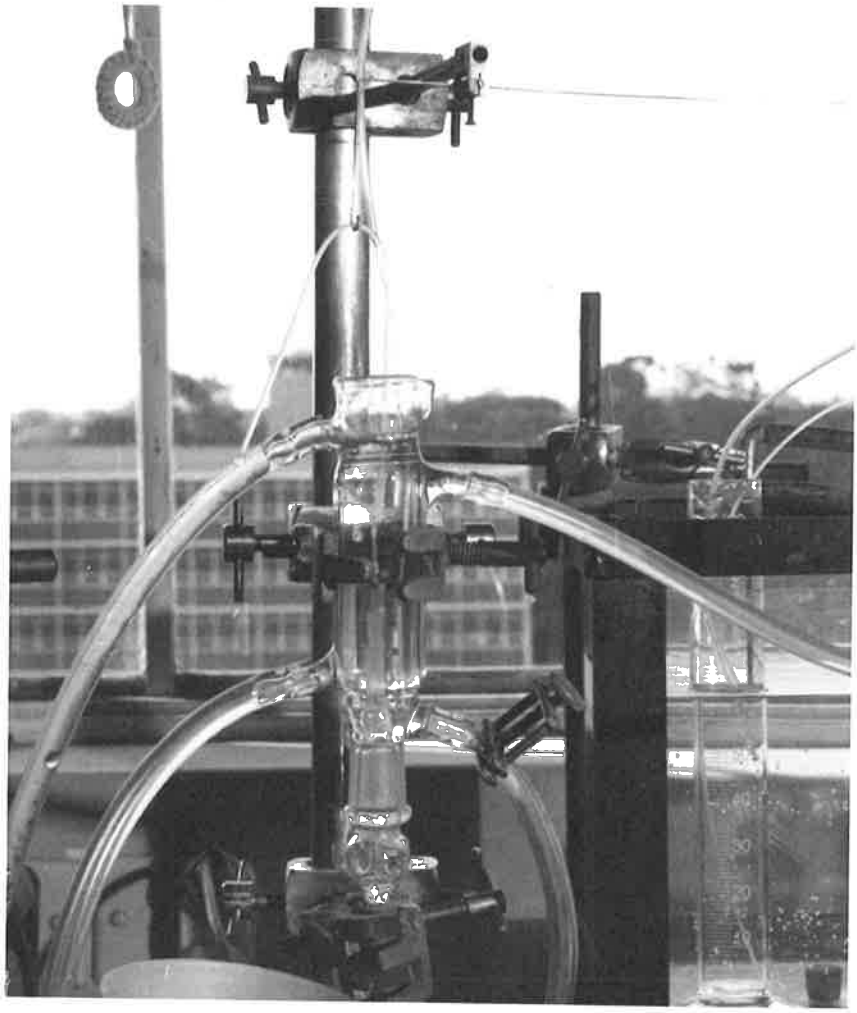
2. The constant volume pump (Appendix Fig. 1) was designed by O. Saxby, Department of Pharmacology, Oxford University, and was manufactured in the Medical School Workshop, University of Adelaide.

3. Heated water was circulated through the warming coil of the perfusion apparatus by means of a Braun circulating pump (Thermomix model) with a thermostat.

4. The organ bath (Appendix Fig. 2) containing the isolated and cannulated artery, had an inner and outer wall in between the two layers of which, heated water, maintained at 37^{o}C, was circulated by means of the Braun pump. The bath had an opening at the top and there was a removable glass plug inserted at its lower end, through which the perfusion fluid entered the artery. The volume of the various organ baths employed varied between 10 and 20 ml and the volume of each bath was measured carefully.



Appendix Fig. 1 The constant volume roller pump used for the ear artery perfusion experiments.



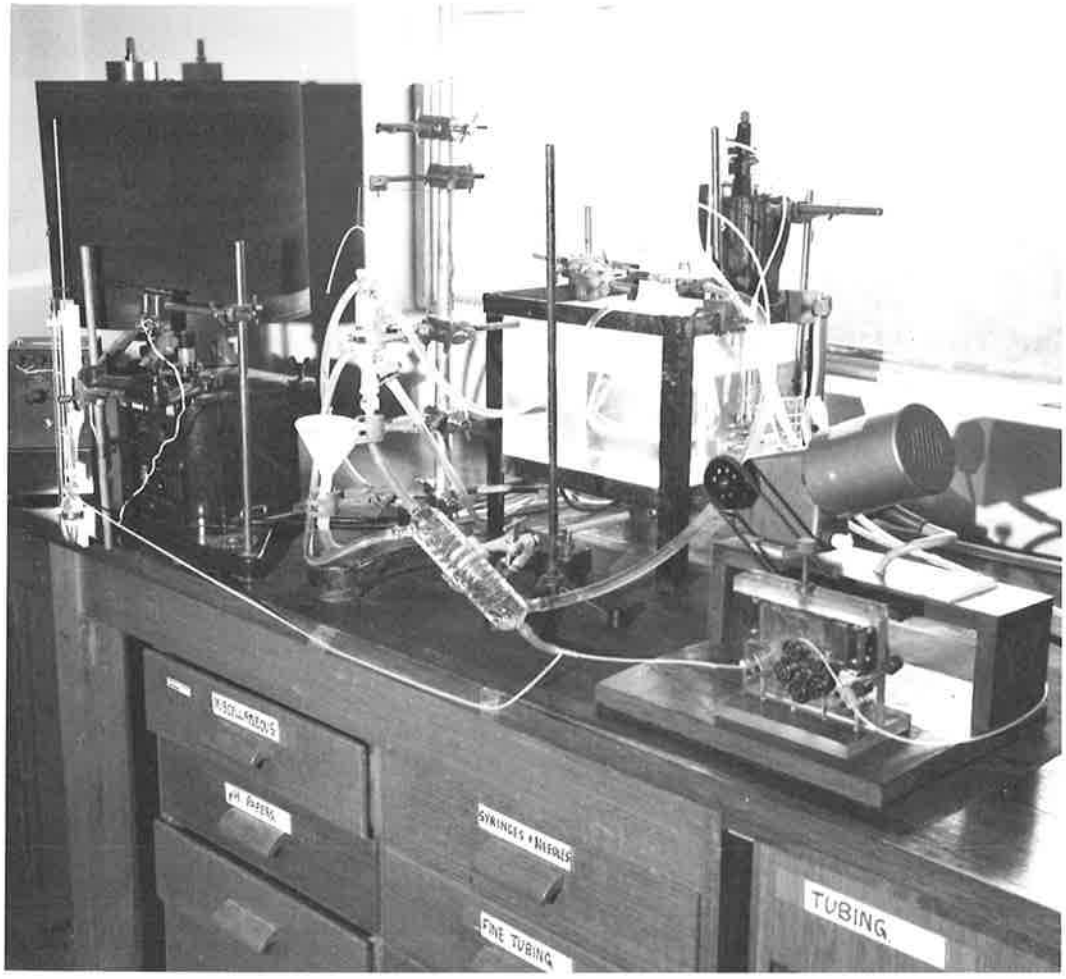
Appendix Fig. 2 Organ bath employed for perfusion of the ear artery. The volume of this particular bath is 17 ml. A double-cannulated artery is seen, with the lever exerting a constant tension on the artery via the distal cannula.

5. In most experiments, pressure changes in the artery were measured via a Palmer mercury manometer (Condon model) recording on a Palmer Kymograph drum (model Super-ten) using a 12 inch (30.5 cm) diameter drum. In some of the noradrenaline sensitivity experiments in Chapter 5, pressure changes were recorded by means of a Statham pressure transducer and a Rikadenki multi-pen recorder (model B-241).

6. Field electrical stimulation was provided by a Grass stimulator (model S4) employing two platinum electrodes.

7. The usual arrangement of the perfusion apparatus used in most experiments is shown in Appendix Fig. 3. In some of the experiments in Chapter 5, intraluminally-applied noradrenaline was given at a slow rate of 1 ml/min by continuous injection into the rubber tubing of the main perfusion circuit immediately below the organ bath. The noradrenaline solution was pumped from a reservoir in the water bath by means of a second roller pump and a warming coil was similarly inserted in this subsidiary circuit before the solution reached the rubber tubing below the organ bath. This rubber tubing was also clipped below the site of the injection needle for the period of the infusion, in order to stop back-flow and thus dilution of the noradrenaline solution in the main perfusion circuit.

8. The perfusion solution employed in all ear artery perfusion experiments was Krebs bicarbonate solution and its composition was as follows:



Appendix Fig. 3 The arrangement of the apparatus used for the majority of the ear artery perfusion experiments. The carbogen cylinder is not shown.

	grams/litre
NaCl	6.9
KCl	0.35
$CaCl_2$	0.28
$MgCl_2$	0.10
$NaHCO_3$	2.1
KH_2PO_4	0.16
Glucose	1.0

Saturation of this solution with 5% carbon dioxide in oxygen reduced its pH from 7.8 to 7.4.

9. In the experiments relating to kinetics of the noradrenaline response in Chapter 5, it was necessary to standardize the method of removing the extraluminal drugs from the fluid bathing the artery in the organ bath so that accurate recovery times from constrictor responses could be measured. This was achieved by employing a volume of Krebs bicarbonate washout solution in each case that was 3 times the volume of the respective organ bath.

B. Noradrenaline fluorescence histochemistry:

1. The freeze dryer employed was a Thermovac model FD-3. The pump was not provided with a gas ballast. This apparatus was not specifically designed as a small tissue freeze dryer and the freezing chamber had a capacity of several litres. No dessicant was employed in the chamber.

2. Paraformaldehyde powder (Merck) was stored (in 5 gram amounts) over 34% v/v sulphuric acid at a relative humidity of 70%. The acid was changed every 2 weeks, irrespective of the quantity of formaldehyde used.

3. The apparatus used for vacuum infiltration was manufactured by the National Appliance Company and was operated using a water vacuum.

4. Tissue sections were cut routinely at 7 microns by means of a Leitz model 1212 microtome.

5. The tissue sections were examined by fluorescence microscopy employing a Leitz microscope with a dry dark field condenser. Fluorescence was obtained by means of an HBO 200 W mercury vapour lamp using a 3 mm Schott BG 12 excitation filter and a 510 millimicron barrier filter. All artery sections were photographed on examination by a Leica camera with microscope and exposure meter attachments. Photographic exposures varied between 10 and 30 seconds, using Kodak Photoflure film which was developed in Ilford ID2 developer.

C. Monoamine oxidase histochemistry:

1. Fresh frozen artery sections of 10 to 20 microns in thickness were cut in a Cryo-cut Cryostat microtome (AO model 830-C) at a temperature of $-25^{\circ}C$.

2. The sections were examined and photographed under the

Leitz microscope using Ilford Micro-file film type 5669, which was developed in Ilford PQ developer.

D. Venous occlusion plethysmography:

1. The water-filled plethysmograph employed is shown in Appendix Fig. 4.

2. The venous occlusion cuff on each wrist was inflated to a constant pressure of usually 60 mm of mercury and subsequently deflated three or four times each minute with air from a compressed air cylinder through two reducing valves. A sequence timer (Paton Industries, Adelaide) which operated solenoid inlet and outlet valves, was set to function automatically so that usually periods of inflation varied from 10 to 14 seconds (Appendix Fig. 5).

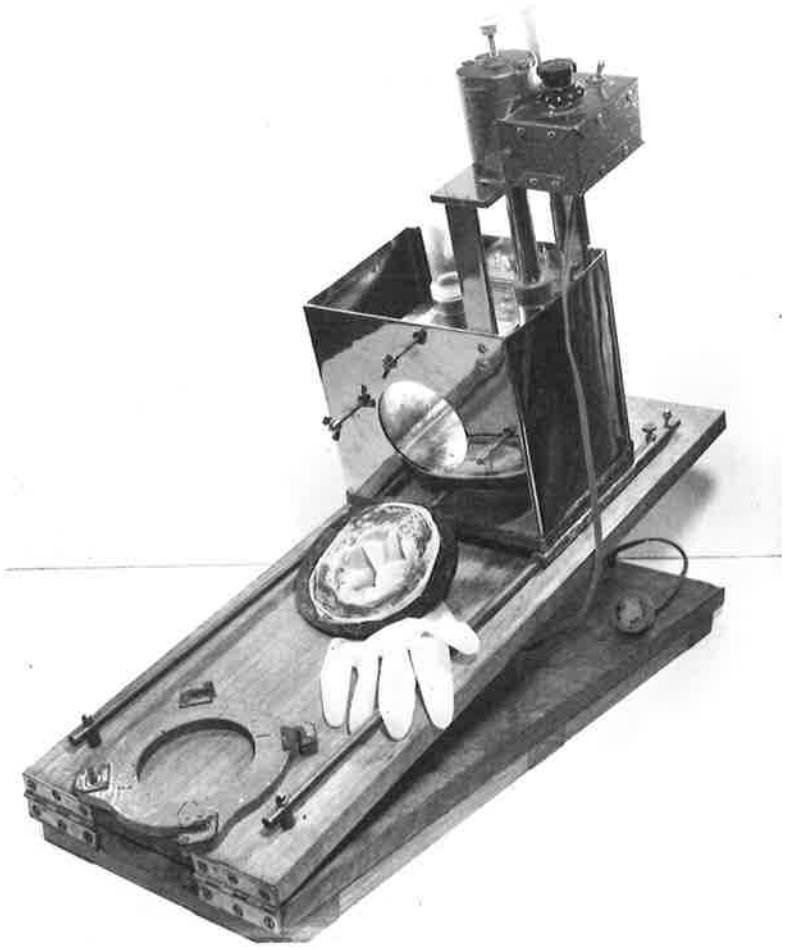
3. The arrangement of the apparatus and the position of the subject for bilateral hand plethysmography is illustrated in Appendix Fig. 6.

4. Appendix Fig. 7 shows the position of the intra-arterial needle which was attached to the controlled perfusion apparatus by fine polythene tubing.

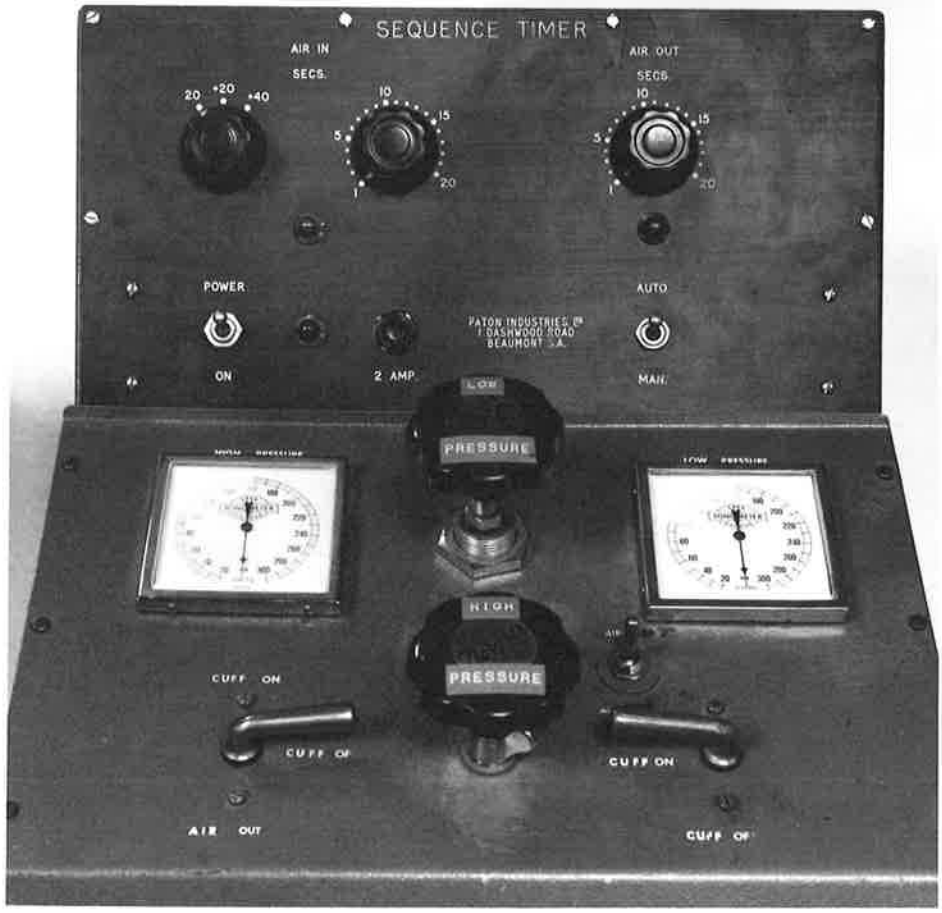
5. The method of determining the hand blood flow values from the kymograph tracing is shown in Appendix Fig. 8.

E. Miscellaneous:

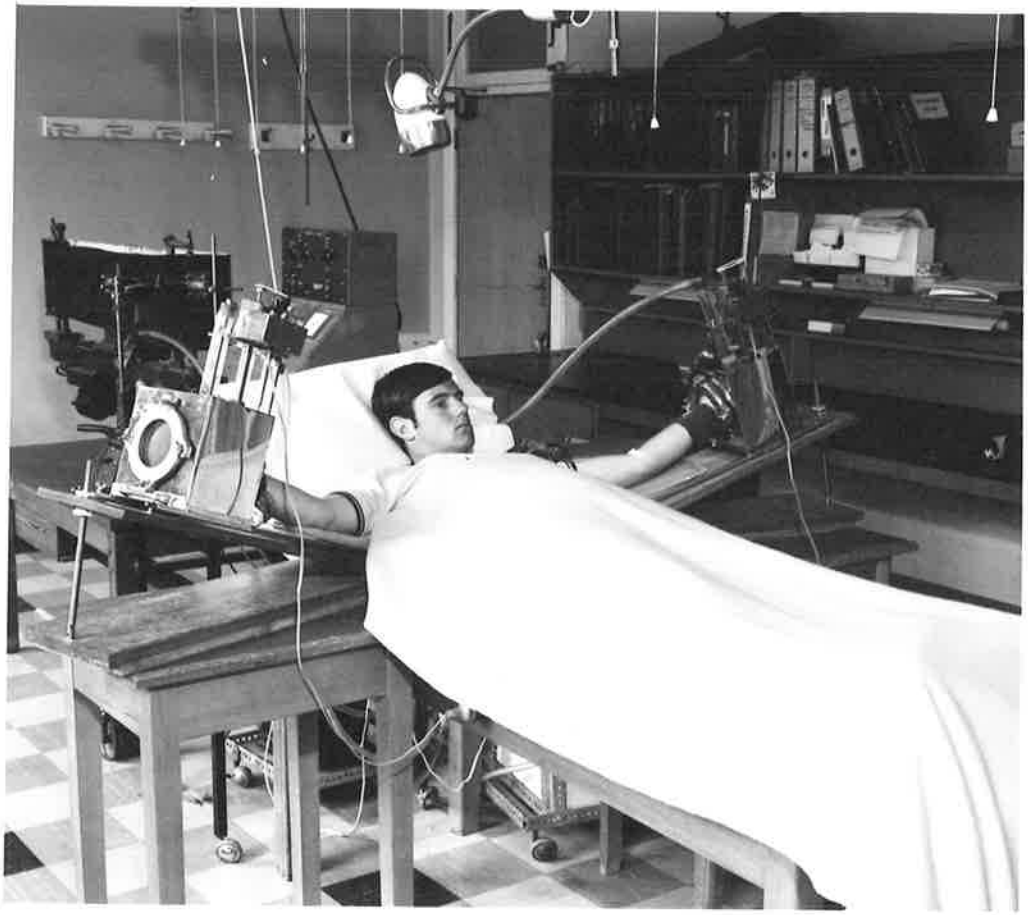
A Beckman (model E350B) pH meter was employed.



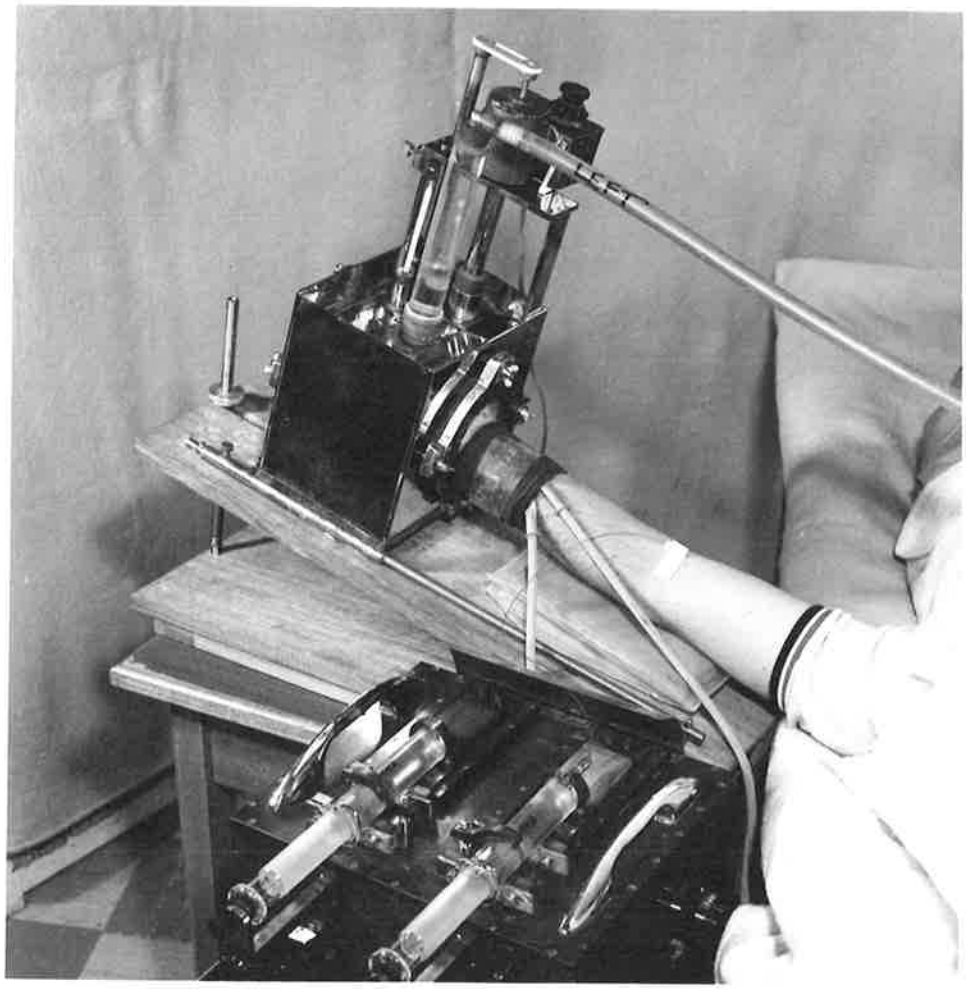
Appendix Fig. 4 The water-filled, temperature-controlled plethysmograph with the rubber glove and inner sealing plate used for hand plethysmography.



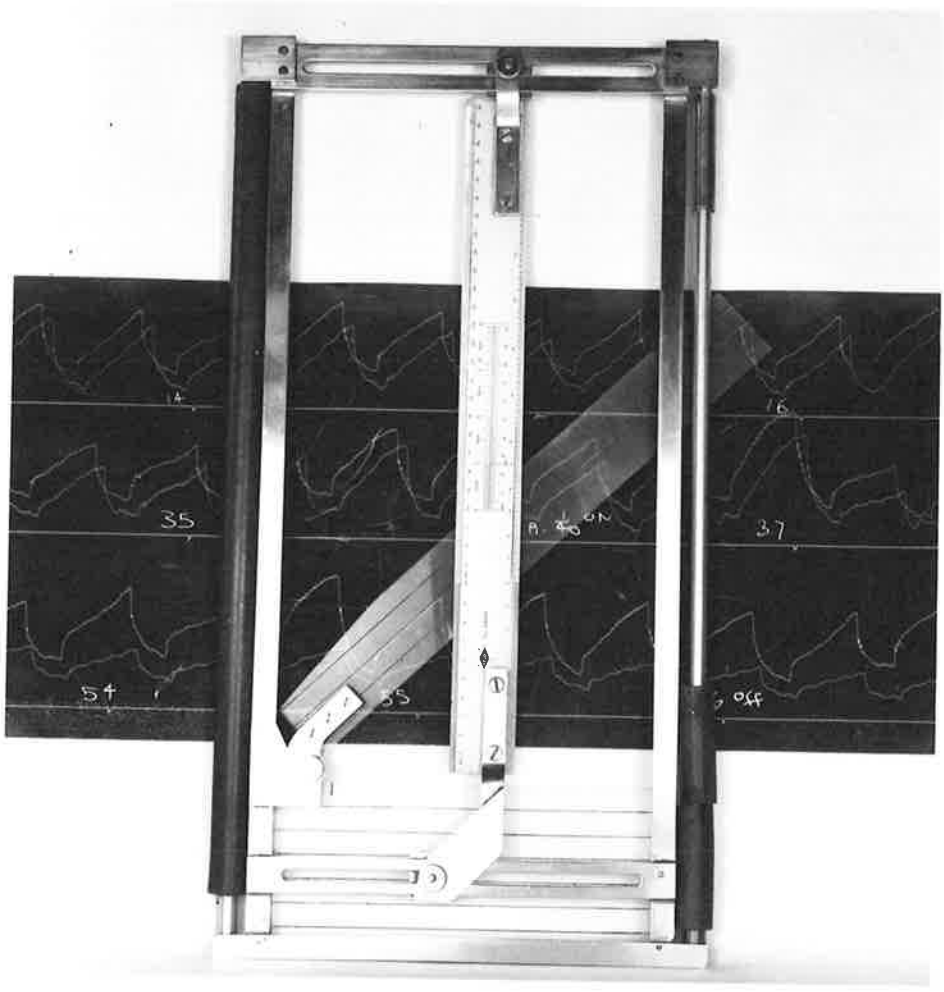
Appendix Fig. 5 The sequence timer used during venous occlusion plethysmography to automatically inflate and deflate the pneumatic collecting cuffs with air from a compressed air cylinder, which is not shown in this photograph.



Appendix Fig. 6 The general arrangement of the apparatus with a subject undergoing bilateral hand plethysmography. The kymograph, sequence timer, pneumatic collecting cuffs and hand plethysmographs are shown. The compressed air cylinder is not visible.



Appendix Fig. 7 Hand blood flow measurement, demonstrating the intra-arterial needle attached by a polythene connection to the controlled infusion apparatus.



Appendix Fig. 8 The apparatus used for measurement of hand blood flow is shown superimposed on a kymograph tracing obtained from a typical venous occlusion plethysmography experiment.

DRUGS EMPLOYED

The dose of each drug was expressed as the weight of the salt except in the case of iproniazid, nialamide, noradrenaline and reserpine in which the weight of the base was used.

Atropine sulphate (British Drug Houses)

Bretylium tosylate (Darenthin, Burroughs Wellcome)

Cocaine hydrochloride (MacFarlane-Smith)

Ephedrine hydrochloride (D. G. Bull)

Histamine acid phosphate (Koch-Light)

Iproniazid (Roche)

Methylamphetamine hydrochloride (Methedrine, Burroughs Wellcome)

Nialamide (Pfizer)

Nitro-blue tetrazolium (Ciba)

Noradrenaline bitartrate monohydrate (Koch-Light) - animal experiments

Noradrenaline bitartrate monohydrate (Levophed, Winthrop) - human experiments

Phentolamine methanesulphonate (Regitine, Ciba)

Reserpine (Serpasil, Ciba)

Sodium sulphate (British Drug Houses)

Tetranitro-blue tetrazolium (Ciba)

Tranylcypromine sulphate (Smith, Kline & French)

Tryptamine hydrochloride (Koch-Light)

Tyramine hydrochloride (Koch-Light)

PREPARATION OF DRUGS

Animal experiments:

1. Noradrenaline bitartrate was dissolved in ascorbic saline (ascorbic acid in 0.9% w/v sodium chloride to form a final concentration of 1 in 10,000). Further noradrenaline dilutions were also made with ascorbic saline, the pH of which had been adjusted to the value 5.5 by the addition of 0.5 N sodium hydroxide solution.

2. Nialamide (60 grams) was dissolved, with gentle heat, in 25 to 30 ml of water and was then added to warmed carbogenated Krebs bicarbonate solution so that the final volume was 600 ml. This produced a nialamide concentration of 100 µg/ml.

3. All other drugs were made up in 0.9% w/v sodium chloride solution.

Human experiments:

1. All solutions were sterile and were made up in sterile 0.9% w/v sodium chloride solution.

2. Ascorbic acid was added to the noradrenaline solutions so that the final concentration was 1 in 50,000.

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