# ADRENERGIC MECHANISMS IN RABBIT GINGIVAL TISSUES

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by

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Contents

## SUMMARY

This study was undertaken to ascertain:

(a) the presence and identity of sympathetic neurotransmitters in gingival tissue in rabbits, and

(b) some aspects of the nature of the uptake and metabolism of exogenous $^{3}$HNA in these tissues. Segments of rabbit central ear artery were included for comparative purposes.

1. Both rabbit gingival tissue and dental pulp were assayed for the presence of endogenous noradrenaline (NA), adrenaline and dopamine; all three catecholamines were found to be present, with NA being by far the most prevalent. Although there were no differences between the amine levels in maxillary compared to mandibular pulps, there were regional variations in the gingival tissues. NA levels were depleted following administration of reserpine or superior cervical ganglionectomy.

2. Gingival tissues from four different sites were incubated in $^{3}$HNA of different concentrations and for different time periods and the kinetics of uptake were observed. The pattern was found to be similar to that of the rabbit central ear artery but the capacity to take up $^{3}$HNA differed widely between tissues. For the gingival tissues, the capacity of the uptake system reflected the endogenous NA levels, i.e. palatal gingiva had the highest content of endogenous NA and the greatest uptake of exogenous NA and buccal gingiva the lowest endogenous level and lowest uptake capacity.

3. Further experiments were undertaken to clarify the pattern of metabolism of $^{3}$HNA in gingival tissues. In one series of experiments cocaine was used to inhibit neuronal uptake of $^{3}$HNA, and in another nialamide was used to inhibit the action of monoamine oxidase.

It was found that inhibition of neuronal uptake and repression of monoamine oxidase activity had the expected result of diminishing the deaminated metabolites, but did not produce the concommitant increase in O-methylated products as occurs in the rabbit ear artery. In fact, cocaine depressed the formation of normetapephrine, the major O-methylated metabolite in buccal and lingual gingiva. This might imply that catechol-O-methyl transferase (COMT) is situated intraneuronally in these tissues, but could reflect the presence of a cocaine-sensitive non- neuronal compartment containing COMT. If this is true, then it is possible that the differences between the gingiva removed from different sites noted above are related to the size and distribution of the vascular tissues in each.

4. Thus, while some similarities exist between the metabolism of NA in gingival tissue and the rabbit ear artery (as an example of a small muscular artery) there are also some significant differences between the two tissues, the chief of which would appear to be a cocaine sensitive extra-neuronal compartment in gingival tissue containing COMT. The site and nature of this compartment requires further investigation.

## DECLARATION

I declare that this thesis contains no material which has been accepted for the award of any other degree or diploma in any University, and to the best of my knowledge contains no material previously published by another person, except where due reference is made in the text.

INES PARKER

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# CHAPTER 1

# INTRODUCTION

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## SYMPATHETIC NEUROTRANSMISSION

As early as the 1930's workers such as Cannon, Folkow and Gaddum produced evidence that the neurotransmitter in sympathetic nerves was noradrenaline (NA).

Von Euler and Hillarp added further information about the distribution of NA both in the axons and terminals of post-ganglionic sympathetic nerves, and the apparently particulate nature of its storage (Euler 1954, 1958, Euler and Hillarp 1956, Hillarp 1959).

While Hillarp had to some extent clarified the functional organization of the autonomic innervation using his methylene blue methods, it was not until Falck developed formaldehyde vapour fluorescence techniques that a large number of organs was investigated, and more direct evidence of the organization of the adrenergic innervation was provided (Falck et al. 1962), Norberg and Hamberger, 1964, Falck and Torp, 1962). These workers described the sympthetic adrenergic axons as ending in systems of fine, branching, varicose terminals which form networks in close association with effector cells, the brightly fluorescing varicosities containing NA storing granules, a description which closely matched Hillarp's (1959). This visual demonstration of adrenergic neurones caused some excitement and was considered to support von Euler's earlier postulation that NA was contained in discrete storage particles concentrated in the terminals of sympathetic neurones (Euler, 1954, 1958).

## Adrenergic Plexus

Falck (1962), and Norberg and Hamberger (1964) described the adrenergic nerve plexus surrounding blood vessels, as did Waterson and Smale (1962). They noted that in both large and small arteries the adrenergic nerves formed a meshed network or plexus, of variable density, this plexus being located between the adventitia and media of the vessel, surrounding the media like a fibrillar sleeve but generally not penetrating into the muscle cells of the media, although some vessels do exhibit fluorescence in the media. Such a network was also described in association with veins, but this was usually much sparser.

A considerable variation in plexus and node density occurs in relation to different vessels (Bevan and Su, 1973). For example, in the ear artery of the rabbit, the plexus is 2.6 times thicker than that in the aorta, and the number of nodes per unit surface area is in the ratio of 4:1.

## Adrenergic Storage Granules

Molinoff (1974) described three types of membrane limited vesicles in nerve terminals:

1. Large, dense-cored vesicles thought to be synthesized in the cell body (Vanhoutte, 1978).
2. Small, dense-cored vesicles which can be recycled and refilled after exocytosis. Vanhoutte (1978) stated that the adrenergic vesicles emptied only part of their content of NA on fusion with the cell membrane, the exocytotic sequence being terminated upon restoring the continuity of the vesicle membrane, the resulting

vesicle being smaller than originally. This smaller vesicle nevertheless has the capacity to take up NA and dopamine, and to convert the latter to NA. It can therefore be refilled with NA and recycled for exocytotic function.

3. Small, electron-lucent vesicles. These would seem to be vesicles empty of NA and can also be recycled to take up and store NA once again. Electron-dense cored vesicles assume this appearance after, for example, treatment with reserpine, which depletes them of their NA stores.

Uptake of NA into the granules is energy dependent and requires $Mg^{++}$ and ATP whereas uptake into the neuronal cells requires Na and ATP. Components of the vesicle membrane could supply the energy required for transport across the membrane as well as for maintenance of high concentration gradients, for example, an electron transfer chain and ATPase (Casey et al., 1977).

Neuromuscular Cleft

The average distance between a node and vascular muscle varies from vessel to vessel. For example, in rabbit pulmonary artery a diffusion path of 4 microns is common, whereas in rat mesenteric arterioles, this may be as small as 0.1 micron. The cleft becomes narrower as the size of the vessel decreases, but separations of less than 0.1 micron, such as those occurring at skeletal neuromuscular junctions, have rarely been reported (Bevan and Su, 1973). The smaller the neuromuscular distance, the more closely it resembles a true synapse and the greater the concentration of intra- synaptic transmitter. The significance of the cleft size in relation to neuronal uptake is discussed later.

Adrenergic Receptors

Ahlquist (1948) defined the adrenergic receptor as "that part of certain effector cells that allows them to detect and respond to epinephrine and related compounds. The receptor can be described only in terms of effector response to drug application".

The distinction between the two different adrenergic receptors, alpha receptors and beta receptors, was made on the basis of difference in potency and susceptibility to different blocking agents. The terms alpha and beta were selected only because "excitatory" and "inhibitory" did not adequately describe these receptors (Axelsson, 1971).

There followed several years of intensive research into the nature of these receptors by workers such as Kirpekar, Starke, Stjärne and Langer. As well as publishing the results of specific studies, Langer wrote a number of review articles summarizing the available information on the pre-synaptic regulatory mechanisms for NA release (Langer, 1974; Langer et al., 1975a).

It was apparent at this time that not only were there alpha and beta receptors in association with adrenergic nerves and their effector organs, but that there were pre and post-synaptic receptors; the difference between the two was not only anatomical but also pharmacological, in that their responses to receptor agonists and antagonists were different (Langer, 1974; Langer et al., 1975a).

Adrenergic receptors have been the subject of numerous studies in the last decade, but these will not be discussed as they are not specifically relevant to this project.

Release of NA

There is a spontaneous leakage or efflux of NA from storage granules, but most of this is rapidly inactivated by cytoplasmic monoamine oxidase (MAO).

Active release or "secretion" of NA is brought about by nerve stimulation or depolarization, and results in quantal discharge of NA from the storage vesicles in adrenergic nerve terminals. The two processes appear to be quite distinct and only the latter is calcium dependent, exocytotic in nature, and subject to receptor mediated local control (Stjärne, 1979).

The exact mechanism of NA release from vesicles is still unclear, but the current view is that exocytosis occurs by a temporary fusion of the vesicular membrane with the cell membrane, the vesicle membrane subsequently being retrieved and reconstituted (hence the radiolucent vesicles) and then refilled with newly formed NA (hence the small radio-dense vesicles). It is also believed that the small radio-dense vesicles are the main source of readily released NA from the storage pool, rather than the larger vesicles containing "older" NA.

Noradrenergic release mechanisms provide an area of catecholamine research which has stimulated much work by biochemists. This is ably summarized in reviews such as that of Casey et al. (1977) and Smith

(1979). Much of the information gained from these studies is related to identifying the constituents of vesicles, and to investigating the hypothesis that NA release from nerves occurs by exocytosis. The weight of evidence from biochemical studies supports this hypothesis (Smith, 1979).

Much descriptive evidence exists (Stjärne, 1981) suggesting that the amount of NA released at a nerve terminal subsequent to a nerve impulse is very variable and controlled by the interplay of a variety of factors. The frequency and variety of these local control mechanisms suggests that they are physiologically important, but their purpose is as yet unclear.

## UPTAKE AND STORAGE OF NA

In 1976, Paton collected the available information on NA uptake in a series of articles and reviews by a number of authors (Paton, 1976). It had been clearly shown that NA accumulated in adrenergic neurones as the result of two separate processes, the first being passage of the amine across the cell membrane of the neurone (uptake) and the second being accumulation of the amine within storage granules (storage). NA could also be taken up and loosely bound by extra-neuronal structures such as smooth muscle cells, collagen and elastin (Avakian and Gillespie, 1968).

The uptake of NA into neurones has been shown to be an active process which is dependent on such factors as temperature, $Na^+$ and $K^+$, and which can be inhibited by a number of drugs or metabolic inhibitors. It is not always possible to distinguish whether the inhibition occurs

by interference with the uptake process or the storage process. For example, reserpine can deplete neuronal NA stores - if neurones depleted by reserpine are exposed to NA, uptake will occur, but with little or no accumulation of NA in the neurone. If intra-neuronal monoamine oxidase is also inhibited, then accumulation of NA will occur, thus indicating that reserpine interferes with the storage process rather than the uptake process.

Neuronal re-uptake of NA appears to be very significant in the termination of action of NA released from noradrenergic neurones. This is illustrated by the potentiating effect of drugs which block the uptake process, thus allowing prolonged action of NA on an effector organ. Uptake of NA by smooth muscle is another mechanism by which the activity of NA might be terminated. In this instance NA is bound only very loosely to smooth muscle and readily diffuses out of the cell; hence it is the intracellular degradation by catechol-O-methyl transferase (COMT), and to a lesser extent MAO, which causes inactivation of the NA. If enzyme activity is inhibited, the constrictor response of smooth muscle cells may in fact be prolonged, since the intracellular NA acts on receptors of the cell membrane. If receptors as well as enzyme activity are blocked, this prolonged constrictor response is abolished (Gillespie, 1976). Smooth muscle uptake becomes of greater importance in tissues where neuromuscular cleft width is too wide to permit maximal effect of neuronal uptake, as for example, in larger blood vessels.

Smooth muscle uptake is an intracellular, saturable process, non-steriospecific and not particularly specific in that it has greater affinity for isoprenaline and adrenaline than for noradrenaline, and will also transport 5-hydroxytryptamine and histamine (Iversen, 1973). Intracellular concentrations as great as four times the extracellular concentration of amine can be achieved, the uptake process being a bidirectional, energy requiring, sodium ion and temperature dependent form of facilitated diffusion. This uptake is inhibited by phenoxybenzamine and other alkylamines, by normetanephrine, steroid hormones and cold, but is unaffected by cocaine, for example, which inhibits neuronal uptake.

Another extraneuronal uptake mechanism is exhibited by the connective tissue components, collagen and elastin (Avakian and Gillespie, 1968). Unlike neuronal and smooth muscle uptake, this process seems to be unaffected by drugs, temperature, ionic or pH changes. However, Powis (1973) found that tetracyclines, which bind to collagen, inhibit the uptake of NA by collagen and elastin. He found that in tissues with a high connective tissue content, such as rabbit ear artery and rat tail, oxytetracyclines could potentiate the response to NA 10 fold and to nerve stimulation, 6 fold. Although blocking neuronal and smooth muscle uptake in these tissues also potentiated the response, the effect of blocking connective tissue uptake was much greater, suggesting that this mechanism might be of considerably greater significance in some tissues than was previously believed.

METABOLISM OF NA

When released NA is taken up by the neurone, much of it is incorporated again into the storage vesicles, but some is metabolized, this process being summarized in Fig. 1. Intraneuronal NA is metabolized by the enzyme MAO, resulting in the deaminated metabolites, 3,4 dihydroxyphenylglycol (DOPEG), and 3,4 dihydroxy-mandelic acid (DOMA); so too is the NA which diffuses into the neuronal cytoplasm as a passive efflux from the storage granules. While a small proportion of the O-methylated metabolites resulting from the action of COMT do appear in association with intraneuronal metabolism, MAO is the dominant enzyme at this site (Langer, 1970; Langer et al., 1972; Langer and Enero, 1974; de la Lande et al., 1970; de la Lande and Jellett, 1972). Extraneuronal uptake, on the other hand, may result in a variable proportion of O-methylated metabolites and the deaminated metabolites normetanephrine (NMN), 4-hydroxy, 3-methoxyphenylglycol (MOPEG), 4-hydroxy,3-methoxymandelic acid (VMA).

COMT is considered to be the predominant enzyme in the extraneuronal compartment, but tissue and species variability exists - for instance, Fiebig and Trendelenburg (1978) noted equal proportions of deaminated and O-methylated metabolites in rat heart, whereas Graefe et al. (1975) did not observe any significant amounts of deaminated metabolites formed extraneuronally in cat heart.

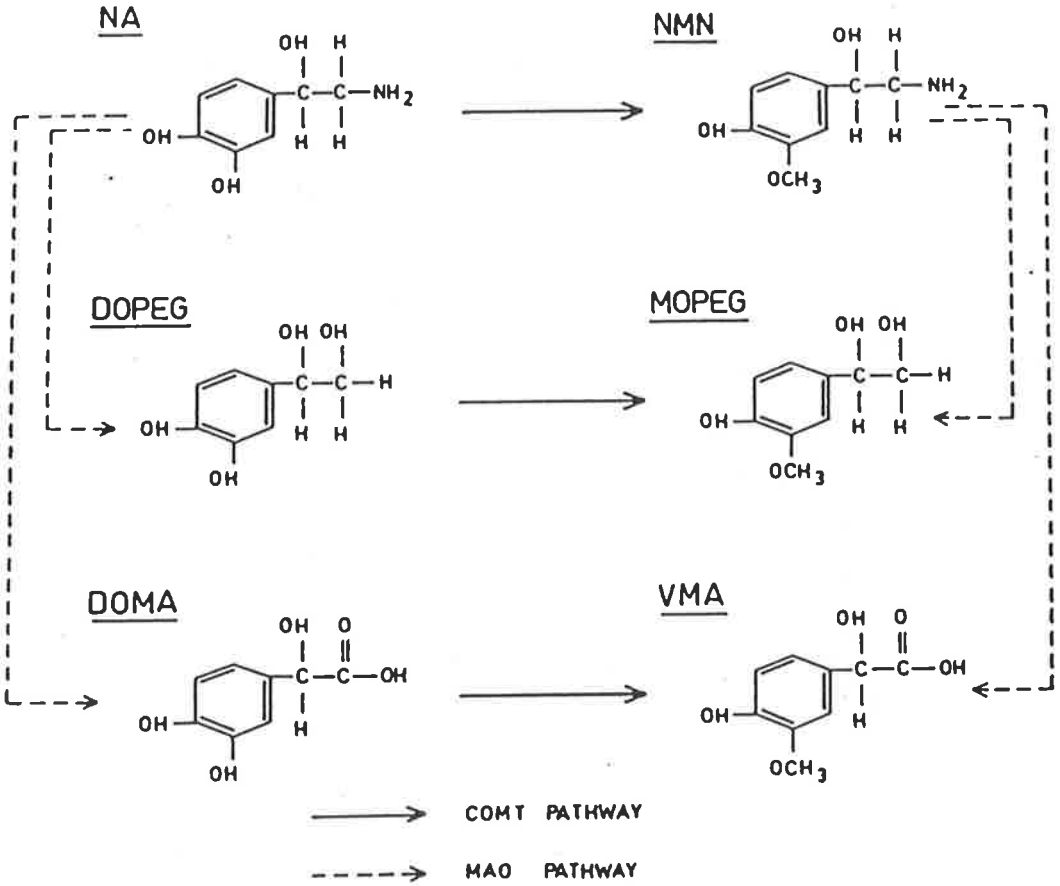


*FIGURE 1* Metabolic pathways for NA.

The diagram indicates the structure of NA and the structures of its deaminated and O-methylated metabolites. The symbols used represent:

NA - noradrenaline
NMN - normetanephrine
DOPEG - 3,4-dihydroxyphenylglycol
MOPEG - 4-hydroxy,3-methoxyphenylglycol
DOMA - 3,4-dihydroxymandelic acid
VMA - 4-hydroxy,3-methoxymandelic acid.

A number of tissues (cat nictitating membrane, rat heart, rabbit aorta, rat sub-maxillary gland, dog saphenous vein, rabbit ear artery) display responses and metabolite patterns which suggest that extraneuronal uptake and O-methylation make a significant contribution to inactivation of NA in these tissues (Trendelenburg, 1978; Head et al., 1975). In such tissues, inhibition of extraneuronal uptake has the same potentiating effect or response to NA as inhibition of COMT.

Although small quantities of O-methylated metabolites appear to result from intraneuronal metabolism of NA, the existence of intraneuronal COMT is considered doubtful. It is possible that the O-methylated metabolites may form extraneuronally as the result of some leakage of deaminated metabolites from the neurone (Fiebig and Trendelenburg, 1978).

Levin and Wilson (1977) separated adventitia and media in rabbit aorta, and investigated NA metabolism in these separate compartments. They found that both adventitia and media contained COMT and MAO activity, but there were some differences in the response of MAO at different sites to enzyme blocking drugs. On this basis it was considered that the isolated adventitia contained mainly type A MAO, while the media contained type B MAO.

Jacobowitz (1972) noted the presence of both MAO and COMT in fibroblasts from guinea pig ventricle, and at the Fourth Meeting on Adrenergic Mechanisms in Portugal, 1980, Azvedo et al. reactivated interest in fibroblasts with a poster demonstrating the close association of fibroblasts with adrenergic fibres in dog mesenteric

arteries and another reporting on the capacity of fibroblasts in dog thoracic duct to take up catecholamines.

In the discussion relating to these posters, de la Lande pointed out that he had observed a similar morphological appearance in photographs of rabbit ear artery, in which fibroblasts could be seen interposed between adrenergic nerves and smooth muscle cells (Hume and Waterson, 1978).

Further information by Starke indicated that work in his laboratory had demonstrated effects on fibroblasts from rabbit spleen, by beta and alpha adrenergic receptor agonists, these agonists stimulating the production of prostaglandins and a consequent increase in the formation of cyclic AMP.

There is therefore interesting evidence that fibroblasts are to be found in close association with adrenergic nerve terminals, that they contain MAO and COMT and also have alpha and beta adrenergic receptors. The speculation is that fibroblasts may be influenced by sympathetic mechanisms, and/or may form part of the extraneuronal uptake system.

## NEUROMUSCULAR TRANSMISSION IN BLOOD VESSELS

Because the sympathetic nerve plexus seldom penetrates into the media (Bevan and Su, 1973), all but the outermost layer of smooth muscle cells are some distance from the nerve terminals, and the assumption is made that excitation occurs either by chemical stimulation of the

first layer of cells which then propagate excitation by, for example, electrical transmission through adjacent cell contact, or by diffusion of the transmitter through the smooth muscle layers. Bevan et al. (1969) suggested that both these explanations were valid, some vessels exhibiting one and some the other mechanism.

Bevan and co-workers looked at the elastic arteries of the rabbit (aorta and pulmonary artery) in which they considered propagation of smooth muscle contraction to occur in direct response to transmitter NA as it diffused through the media. They found that of the NA released at the adventitio-medial junction by nerve impulses, about 80% was taken up by the neurones, and less than half of the balance entered the media, initiating contraction of successive layers of smooth muscle cells as it slowly diffused through the media in ever decreasing concentration. Having penetrated the media, NA is taken up and loosely bound by extraneuronal structures such as smooth muscle cells, collagen and elastin (Avakian and Gillespie, 1968). This uptake mechanism, together with enzymatic degradation by monoamine oxidase (MAO) and catechol-O-methyl (COMT) transferase is mainly responsible for the inactivation of transmitter within the vessel wall.

Waterson and de la Lande (1967) pointed out that a number of histochemical studies clearly indicated that the extent of sympathetic innervation of blood vessels varied considerably according to the location and function of these vessels, and where there was necessity of rapid adaptation of blood supply, the sympathetic innervation was correspondingly generous.

## VASOMOTOR CONTROL IN GINGIVAL TISSUE

As regards gingival tissue, very little work has been done in relation to the physiology of its blood supply, but the morphology of the latter has been documented.

Incident light microscopy had been utilized for the direct observation of gingival capillary beds as far back as the 1920's (Kamijo et al. 1964). Forsslund (1959) described not only the morphology of gingival vessels in man, but attempted to investigate functional aspects as well, if rather crudely. For instance, he compared the appearance and numbers of vessels in healthy tissues with those in diseased tissues, he looked at the constancy of the vascular bed, and at the effects of injecting adrenaline and histamine.

In the same year, Staple and Copley (1959) provided a description of the regulation of blood flow in the gingival microcirculation of small laboratory animals: incident light microscopy was utilized to observe the labial gingiva of the mandibular incisor region of the hamster, mouse, rat, guinea-pig and rabbit. They noted that except in guinea-pigs, the loop-like capillary arrangement observed in human gingiva did not occur in these small animals, but that a network of vessels was observed. Staple and Copley were able to observe changes in the callibre of arterioles, opening and closing of exits from main capillary channels into capillary networks in which vasomotor activity appeared to be absent, and opening and closing of arterio-venous anastomoses.

Kamijo et al. (1964) also employed photomicrography of labial gingiva and mucosa to observe the blood supply of these tissues in human subjects. They compared their findings with those of Staple and Forsslund, but made an attempt to classify capillary loop forms according to the number and position of twists in the loop. Kamijo and co-authors reported that the number of vessels per square mm in healthy gingiva was about 60, compared with 45 in diseased gingiva. (These figures were rather higher than Forsslund's 45 and 20 respectively, although the range between individuals was considerable). These results are comparable to those for skin, but considerably higher than the 23 vessels per square mm found in the oral mucosa at the vestibular fold.

In describing the various morphological loop forms, Kamijo et al. also noted that in diseased gingiva not only were the loops more distended, but also more tortuous in form than in healthy gingiva.

Kindlova (1965) used replicas of latex filled blood vessels together with histological sections in describing the gingival and periodontal blood supply of the monkey. She provided a very comprehensive picture of the origins of this blood supply and how they inter-related. The main supply of the periodontal ligament arises from the intra-bony vessels, viz. superior alveolar artery and mandibular artery respectively, while the main supply of the gingiva arises from the palatine vessels and the lingual artery: considerable anastomosis exists between the extra and intra-bony sources of blood supply, particularly at the gingival crest. The basic capillary pattern for the periodontal ligament is a flat network, while that in the gingiva consists of a network with the loop extensions into the connective tissue papillae.

Kindlova divided the blood supply of this region into three zones, based on morphology and source of blood supply:

1. The periodontal membrane.
2. The gingiva facing the oral cavity and extending to the gingival crest.
3. The gingiva facing the tooth and lying coronal to the periodontal membrane.

She suggested that the differences may be due to differences in metabolic requirements related to the anatomy of the tissues; the vessels in the periodontal ligament supplying connective tissue only, while those in the gingiva supply both connective tissue and epithelium.

Hansson et al. (1968) devised a method of viewing the crevicular gingiva from the tooth side by cutting away the tooth, but leaving the gingiva intact and accessible to transillumination and vital microscopy. They studied the appearance of the vessels in both healthy and chronically inflamed gingiva. Their description of the vascular pattern in healthy gingiva is similar to Kindlova's (although her study does not indicate whether the gingival health status of the monkeys used was noted). However, the greater detail of Hansson's observations indicates that there is a narrow avascular zone along the sulcular margin (presumably the epithelial band) and that beneath this is a network with some incidence of loop-like structures, in which the direction of blood flow is essentially parallel to the gingival surface. Beneath this again is a wider region of more widely spaced vessels which are essentially perpendicular to the narrower marginal

plexus. By contrast, the marginal plexus becomes indistinguishable in inflamed gingiva, the vasculature now consisting of wide tortuous capillaries and venules, running perpendicular to and in close relation to, the crevicular epithelium.

Hock and Nuki (1971) modified Hansson's method of directly viewing the free gingiva by transillumination, so that the tissue remained supported by tooth structure, and in addition, was viewed from the oral aspect and not from the crevicular surface. They used this method to view the free gingiva of ferrets, opossums, cats, dogs and rhesus monkeys in which the health of the gingiva was assessed clinically, histologically and by vital microscopy.

These authors observed a correlation between vascular morphology and histopathologic changes, but considered that the vascular changes preceded other observable pathologic alterations. They described five vascular patterns with a gradual progression from a straight vesseled network, to a tortuous loop form. Of particular interest was pattern one, consisting of straight vessels parallel to the gingival margin, and which, according to the authors, was observable only in animals with no prior experience of gingival inflammation. Once inflammation has been present, the vasculature did not return to this straight vesseled form even after resolution of the inflammatory process.

Further studies by the same authors (Hock and Nuki, 1975; Hock, 1975) confirmed that the normal vascular pattern in non-inflamed gingiva was a regular network, but as inflammation occurred, this changed to a convoluted, looped pattern through alterations in width and length of capillaries and post-capillary vessels, as well as twisting, looping and spiralling of marginal capillaries.

While previous studies described the origin and macroscopic morphology of the gingival and periodontal blood supply, workers such as Mohamed et al. (1973) studied the ultra structure of gingival vessels in rabbits, using the electron microscope. This study described the fine gingival vessels as capillaries, post-capillary venules, muscular venules and arterioles.

The capillaries consisted of endothelial cells surrounded by a basement membrane. Of particular interest was that these endothelial cells displayed projections into the lumen and also through the basement lamina into the peri-capillary space. About 30% of the observed capillaries also exhibited fenestrations in the endothelial cell layer, these fenestrations generally being covered by a membrane, and most frequently facing the epithelial surface of the gingiva. The authors speculated that these fenestrations therefore played a role in the nutrient transfer from capillary lumen to the overlying epithelium. Surrounding the endothelial cells of the capillary were pericytes or fibroblasts, the former being distinguished from the latter by their closer adaptation to the endothelial cells and the presence of surrounding lamina.

The post-capillary venules differed from the capillaries in that the endothelial cells displayed some overlapping at their junctions and contained more dense granules within their cytoplasm. The basement lamina was observed as a continuous layer outside the more numerous enveloping pericytes.

In the larger, muscular venules, smooth muscle cells replaced pericytes and formed a continuous layer arranged in a spiral fashion, surrounded by a basement lamina.

Small terminal arterioles observed in this study had one or more continuous layers of smooth muscle cells, without the overlapping, spiral arrangement seen in the venules. Bundles of non-myelinated nerves were also seen in association with the arterioles.

Frewin et al. (1971) studied haematoxylin and eosin stained sections of human gingival tissue and found numerous blood vessels, some of which had muscle cell layers four to five cells thick.

Gingival samples from 25 subjects with a wide age distribution were also treated with the Falck formaldehyde method to produce catecholamine fluorescence. The green fluorescence specific to monoamines was seen at the outer border of the smooth muscle layer of blood vessels in some, but not all, of the gingival samples. This fluoresence occurred most frequently and was strongest in the younger age groups; gingival tissues from the older subjects (50+ years) exhibited only weak fluorescence, if any.

The authors interpreted these results as indicating that noradrenergic nerve fibres were associated with blood vessels in human gingival tissue and that the density of these fibres and/or their noradrenaline content decreased with age. The other points of interest noted were that not all muscular vessels demonstrated noradrenergic fluorescence, and conversely, noradrenergic nerves and varicosities were occasionally observed which appeared not to be associated with blood vessels.

The material for this study was obtained at autopsy, but a later study by Waterson et al. (1974) carried out on vital tissue produced similar results.

Verco (1981) developed an interesting technique whereby experimental and control tissues were included in the same tissue section. This was achieved in rats in which unilateral sympathetic ganglionectomy had been carried out one month before sacrifice. The total palatal mucosa was stripped off, treated by the Falck formaldehyde technique to produce catecholamine fluorescence, and sections cut across the full width of tissue thereby providing a comparison of the denervated and control (normal) mucosa in the same section. Catecholamine fluorescence was absent on the sympathectomized side, but brilliantly clear on the normal side. Also of interest was the apparent decrease in mast cell numbers occurring on the denervated side.

Although some work has been done on the sympathetic nerve supply to the pulpal blood vessels, very little has been published on that of gingival vessels.

Edwall and Kindlova (1971) briefly investigated the effect of sympathetic nerve stimulation on the blood vessels in the gingiva as well as the pulp, periodontal ligament and alveolar sub-mucosa in cats and dogs (utilizing the tracer disappearance technique). They found that sympathetic nerve stimulation resulted in a reduction of tracer disappearance rate of up to 90%, this degree of vaso-constriction occurring in the periodontal ligament, but about 70% being the usual reduction for gingiva, pulp and sub-mucosa.

Browne (1965) looked at the effects of adrenaline and histamine on the blood flow of gingiva in rabbits, and at the effects on the corresponding production of gingival fluid. Changes in temperature in gingival tissues were used as indicators of changes in blood flow. He found that intra-arterial injection of adrenaline produced a drop in gingival temperature (i.e. vasoconstriction of gingival vessels) while histamine had the opposite effect. In a similar study, Clarke et al. (1981) looked at the effect of intra-arterial injections of nicotine and adrenaline on gingival blood flow, their hypothesis being that stress (producing adrenaline) and smoking (nicotine) might contribute to the ischaemia of end arteries in gingival tissue, so initiating acute ulcerative necrotizing gingivitis. Using rabbits as the experimental animals, these authors monitored blood flow through temperature changes, and it was found that both adrenaline and nicotine caused marked reduction of blood flow in gingival tissue, in spite of a pronounced rise in systemic blood pressure.

In 1975 Merkel described a cell he observed in epithelial rete ridges, which differed from the surrounding epithelial cells in that it had a more irregular nucleus, clear cytoplasm and was almost always in close association with a nerve axon. Merkel believed these cells to be touch receptors.

During the 1970's a small number of papers were written describing these Merkel cells in human skin, oral mucosa and gingival tissue. (Mustkallio and Kiistala, 1967; Smith, 1970; Nikai et al., 1971; Hashimoto, 1972; Wilgram, 1972; Luzardo-Baptista, 1974; Fortman and Winkelmann, 1977). The cells were described as being larger and less

electron-opaque than surrounding epithelial cells, as well as having a more lobulated nucleus. They were also generally associated closely with a nerve terminal, were situated in the basal layer of the epithelium and contained granules which were considered to resemble adrenergic vesicles. One study claimed that mechanical stimulation of an epithelial region containing a Merkel cell, resulted in a discharge of the afferent nerve (Muskallio and Kiistala, 1967). Another study (Wilgram, 1972) speculated on the possibility that psychogenic factors might lead to the discharge of Merkel cell catecholamines, causing vaso-constriction which could then trigger the dermo-epidermal necrosis associated with aphthous ulcers.

Fortmann and Winkelmann (1977) reviewed the literature on Merkel cells and provided a very detailed description of these cells using electron microscopy. They identified an intra-nuclear rodlet which, they claimed, was characteristic of the Merkel cell and could be used as a reliable means of identifying this cell. They also claimed that an identical rodlet had been described by other authors in neural tissue, and that therefore in their view, the Merkel cell is of neural crest origin and is a specialized neural receptor cell.

This group of studies is largely descriptive in nature, with little evidence to substantiate the speculations as to Merkel cell function. No further work appears to have been published - it would be interesting to see what histochemical techniques might reveal, particularly in relation to the granular vesicles observed in these cells.

It is apparent from the foregoing review that very little research has been carried out to investigate the mechanisms influencing gingival blood flow, and with the exception of the previously mentioned reports by Edwall and Kindlova (1971), none at all in relation to sympathetic mechanisms in this tissue. The following study was therefore undertaken to investigate some of the characteristics of sympathetic innervation in rabbit gingival tissue, with the intention of applying the information gained and the experimental techniques developed, to later investigations of human gingiva.

The specific aims of the project were to ascertain whether the neuro-transmitters associated with sympathetic innervation, namely, dopamine, adrenaline and noradrenaline, were present in gingival tissue, and if present, that these endogenous catecholamines were in sympathetic neurones. Having confirmed that sympathetic innervation and neuro-transmitters exist within rabbit gingiva, further work was undertaken to begin to characterize this system in gingival tissue, since it is well known that sympathetic mechanisms are not only species specific, but also tissue specific. The studies undertaken investigated the uptake and metabolism of exogenous NA in rabbit gingiva, and compared this pattern with that of a small muscular artery, the central ear artery of the rabbit.

# CHAPTER 2

# ENDOGENOUS CATECHOLAMINES

# CHAPTER 2

## INTRODUCTION

As indicated, the first objective of the study was to determine whether the known sympathetic transmitters dopamine (DA), adrenaline (A) and noradrenaline (NA) were present in gingival tissues. In initial experiments, the suitability of the radioenzymic method of Da Prada and Zürcher (1976) and high performance liquid chromatography were investigated using rat and rabbit gingiva.

As the high performance liquid chromatography facilities available were not sufficiently sensitive to detect the very low levels of catecholamines in rat and rabbit gingiva it was decided to use the radioenzymic method. Moreover, the gingival samples obtainable from the rat were very small, and therefore the larger rabbit was selected as the experimental animal. The rabbit provided additional advantages in that the central ear artery could be used as a comparison and also as a means of verifying experimental techniques, since catecholamine pharmacology has been well documented for this tissue. In addition, rabbit dental pulps were included in some parts of this project; this was done partly to provide an interesting comparison between two oral tissues, but also to provide information on rabbit pulps as part of an extended research programme.

## METHODS

Semi-lop eared rabbits were stunned and bled, after which pieces of gingival tissue, dental pulp and atrium were removed.

Gingival tissues were removed from four sites on each side of the mouth, namely, palatal, maxillary and mandibular buccal, and lingual, by excision of a strip of free and attached gingiva adjacent to the molar teeth; the resultant tissue was approximately 3 mm wide and 15 mm long.

All four incisor teeth (2 maxillary and 2 mandibular) were excised whole, then split, and the exposed pulps removed intact.

Finally, the chest cavity was opened with a central incision and a piece of atrium removed from the heart.

Tissue samples were placed in ice-cold Krebs' solution immediately after removal, blotted and weighed, then returned to ice-cold Krebs' solution. Samples were then chopped into 0.5 mm cubes using a McIlwain chopper and placed in glass homogenizing tubes together with 0.5 mls. of 0.3 M perchloric acid containing 5 mM ethyleneglycol-bis-(B-aminoethyl ether) N,N$^1$-tetra-acetic acid and 0.5 mM magnesium chloride (i.e., 0.3 M $HClO_4$ + 5 mM EGTA + 0.5 mM $MgCl_2$). Homogenization was carried out using glass pestles until no discrete tissue pieces were visible. Homogenates were then poured into storage tubes, homogenizers rinsed with another 0.5 mls ($HClO_4$ + EGTA + $MgCl_2$), and this wash removed to the storage tubes with a pasteur pipette.

Homogenates were refrigerated overnight and centrifuged next day. The supernatant was aspirated and placed in fresh storage tubes; in some experiments this was then radio-enzymically assayed the same day, and in others the samples were frozen and assayed within a week as below.

Assay of Endogenous Catecholamines

The simultaneous radioenzymic determination of the tissue content of A, NA and DA described by Da Prada and Zürcher (1976) was the method essentially used to assay the endogenous levels of these catecholamines in rabbit tissues in the present study. This method, with modifications, was validated as a reliable means of assaying the three catecholamines at the levels present in rabbit vasculature, by Head et al., (1982).

After acid extraction of A, NA and DA from the tissues, the catecholamines were converted to their O-methylated analogues (metanephrine, normetanephrine and methoxytyramine respectively) using COMT prepared from mouse liver, in the presence of S-adenosyl-methionene-$^{3}H$ which donates the radioactively labelled methyl group.

The polarity of the O-methylated derivatives was reduced using sodium tetra-phenylborate, after which they were extracted into di-ethyl ether. Following further refinement of the extraction process, the O-methylated products were concentrated by freeze drying and reconstitution in a small quantity of solvent (50 ul methanol/acetic acid).

The products were then separated by spotting onto thin layer Kieselgel chromatography plates which were suspended for 30 min in a solvent consisting of chloroform:methanol:ethylamine (16:3:2). After drying, the separated metanephrine, normetanephrine and methoxytyramine were visualized as fluorescent spots under UV light, cut from the plate and placed in separate scintillation vials. The catecholamine derivatives

were extracted from the gel with hydrochloric acid, toluene-triton scintillant added, and the radioactivity counted using scintillation spectrometry (described later).

All samples were prepared and counted in triplicate, the mean value being compared to the radio-activity levels of standards of known concentrations, from which comparison the levels of endogenous catecholamines were calculated.

Scintillation Spectrometry

The radioactivity (level of $^{3}H$) in all samples was determined by liquid scintillation spectrometry using a Beckman Scintillation Spectrometer. The scintillation medium contained toluene and triton-X100 (2:1 ratio) and the spectrofluors PPO (2,5-diphenyl-oxazole) and dimethyl-POPOP (1,4-bis(2(4- methyl-5-phenyloxazolyl)) benzene) (5.5 $g.l^{-1}$ and 0.17 $g.l^{-1}$ respectively). Quenching was determined for each sample by referring the ratio of counts in two tritium channels to a quench curve determined for the counter using a commercially prepared set of quenched standards. Hence counts per minute were converted to disintegrations per minute and this figure used for all calculations. Each sample was counted for 10 minutes or until pre-determined count levels were attained.

Reserpinized Rabbits

Gingival tissue, pulp and atrium were removed from 5 rabbits which had been injected intra-peritoneally with reserpine (2.5 mg/kg body weight) 16-20 hours prior to sacrifice, and treated as described above. The endogenous catecholamine contents of the tissues were assayed.

Sympathectomized Rabbits

A number of rabbits were anaesthetized with Ketalar (100 mg ketamine hydrochloride and benzethonium chloride, 0.1 mg in 1ml solution) using 2 ml per 1 kg body weight, injected intra-muscularly into the hind leg.

After shaving the neck, a midline incision was made through skin and muscle layers over the trachea, and tissues further separated by blunt dissection to locate the sympathetic cervical ganglion on one side. The ganglion was exposed by blunt dissection and then cut from the trunk both proximally and distally. The incision was closed in 2 layers (muscle and skin) with multiple black silk sutures. The animals were then injected with 0.2 mls penicillin per 1 kg body weight (aqueous suspension of procaine penicillin 300 mg per 1 mg) to minimize the risk of infection.

The animals were sacrificed 2 to 4 days after ganglionectomy and gingival tissues and pulps removed and processed as before. As well as removing a section of atrium, pieces of the central arteries from both left and right ears were removed as comparative tissues. Catecholamine levels were assayed by the radioenzymic method described above.

Drugs

The following drugs were used in the experiments described:

| | |
|---|---|
| Reserpine | Sigma, St. Louis, U.S.A. |
| Ketalar | Parke-Davis Pty. Ltd. N.S.W. |
| Vetspen | Glaxo Aust. Pty. Ltd. Vic. |

## Statistical Tests

Results are presented as the means +/- standard errors of the means. Paired students' t-tests were used to determine the significance of differences between groups of data; differences between groups were considered to be significant when the probability was below the 5% level. Differences between observed results are described in the text as "significant" (that is, $p<0.05$) or "not significant" (that is, $p>0.05$).

## RESULTS

The endogenous catecholamine contents of the various tissues are shown in Table 1; it can be seen that in all tissues the concentration of NA was much greater than DA which in turn, was slightly greater than A. The concentration of NA in the ear artery was greater than that in atrium, the levels in these tissues being much higher than those in the dental tissues. There were no significant differences between the concentrations of DA, A and NA in either the maxillary or mandibular pulps, but there was a significantly higher level of NA in palatal gingiva when compared to either maxillary or mandibular buccal gingiva (paired t-test; $p < 0.01$). Although NA concentration was greater in palatal tissue than in lingual, and the latter was greater than that in buccal, these differences were not statistically significant (paired t-test; $p > 0.05$).

The effects of reserpine on the contents of the three catecholamines in gingiva, pulp and atrium can be seen in Table 2. In most experiments, DA was not detected in the dental tissues, and its concentration had decreased by 80% in the rabbit atrium. Reserpine

appeared to have little effect on the adrenaline levels in these tissues, except that the amine was not detected in three of the four mandibular incisor pulps and had decreased by 78% in the atrium. The NA content in all tissues had decreased greatly.

The effects of cervical sympathetic ganglionectomy on the concentrations of DA and A in the gingival tissues and dental pulps were variable and will not be reported. However, unilateral removal of the superior cervical sympathetic ganglion caused the NA concentration on that side of the animal to decrease by 90 +/- 5% in the palatal gingiva, by 86+/-4% in the lingual and 59 +/- 17% in the pooled maxillary and mandibular buccal tissues. The amount of NA in the dental pulps was decreased by 62 +/- 23% and in the REA by 97 +/- 3%.

The concentrations of DA (144.4 +/- 35.3pmol.$g^{-1}$), A (124.5 +/- 19.8pmol.$g^{-1}$) and NA (11.3 +/- 2.2nmol.$g^{-1}$) for rabbit atria were not significantly different from those indicated in Table 1. (paired t-test; $p>0.05$).

## DISCUSSION

Atria and ear artery segments were included in these experiments as their catecholamine contents have been reported previously (Head et al., 1977, 1982) and it was considered that they could provide standards for comparison with the amine contents of the dental pulp and gingiva.

Table 1. Endogenous catecholamine contents of rabbit tissues ($pmol.g^{-1}$)

| | DOPAMINE | ADRENALINE | NORADRENALINE |
|---|---|---|---|
| GINGIVA (n = 4) | | | |
| PALATAL | 10.3 (0.7) | 7.7 (0.6) | 814.4 (182.5) |
| BUCCAL (max.) | 13.9 (4.5) | 9.1 (2.2) | 476.8 (201.2) |
| BUCCAL (mand.) | 17.5 (3.6) | 7.4 (1.6) | 266.5 ( 75.1) |
| LINGUAL | 17.7 (3.1) | 13.7 (3.5) | 359.8 ( 54.8) |
| PULP (n = 4) | | | |
| INCISOR (max.) | 16.3 (5.6) | 11.4 (3.6) | 917.6 ( 26.0) |
| INCISOR (mand.) | 27.1 (6.6) | 19.6 (5.1) | 785.2 ( 84.7) |
| ATRIUM (n = 5) | 110.7 (15.2) | 87.3 (3.3) | 8.28 (.10)$\times 10^3$ |
| EAR ARTERY (n = 3) | 526.1 (207.2) | 49.7 (15.9) | 14.37(2.92)$\times 10^3$ |

Figures in parentheses represent ± SEM

Table 2. Catecholamine contents of rabbit tissues ($pmol.g^{-1}$) after pretreatment with reserpine (I.P. - 2.5 $mg.Kg^{-1}$)

| | DOPAMINE | ADRENALINE | NORADRENALINE |
|---|---|---|---|
| GINGIVA (n = 3) | | | |
| PALATAL | a | 5.9 (2.1) | 34.72 ( 6.2) |
| BUCCAL (max.) | a | 6.9 (3.5) | 52.1 ( 13.3) |
| BUCCAL (mand.) | a | 17.8 (7.9) | 68.2 ( 24.8) |
| LINGUAL | a | 8.3 (2.0) | 26.7 ( 7.4) |
| PULP (n = 4) | | | |
| INCISOR (max.) | b | 8.9 (3.4) | 269.6 (127.9) |
| INCISOR (mand.) | b | b | 165.4 ( 57.4) |
| ATRIUM (n = 5) | 22.2 (12.2) | 18.9 (8.1) | 166.7 (102.7) |

Figures in parentheses represent ± SEM

a - no dopamine detected in 2 of 3 tissues

b - no catecholamine detected in 3 of 4 tissues

The levels of endogenous NA, DA and A in the rabbit ear artery were similar to those reported by Head et al. (1977) who used the trihydroxyindole fluorescence method to assay the amine content in this tissue. In a later study, Head et al. (1982) assayed the catecholamine content of rabbit heart using a technique based on that of Da Prada and Zürcher (1976) and present results are in accord with the levels reported for NA, but are approximately one half of those for DA and A. This difference may be due to the fact that Head assayed total heart tissue while only atrium was used in this study.

The present study provided evidence that reserpine greatly depletes the amounts of endogenous NA, DA and A in the rabbit heart, whereas removal of the cervical sympathetic ganglion does not. In the REA the levels of DA, A and NA were diminished following surgical removal of the ganglion or pretreatment of the animal with reserpine. The present findings confirm the previous histochemical evidence of de la Lande et al. (1974) that ganglionectomy depleted neuronal stores of NA.

Although the levels of NA in bovine and feline dental pulps have been reported (Nakano et al., 1970; Pohto et al., 1972) it appears that the amounts of catecholamines in these tissues in rabbits have not been documented. The present results emphasize the much higher levels of NA relative to DA and A in dental pulp and the similarity between the levels of the three amines in tissues removed from maxillary and mandibular teeth. Because of the low sensitivity of the techniques used by Pohto et al. and Nakano et al., they pooled pulpal tissue from more than one tooth and/or more than one animal. Moreover, Nakano et al. were unable to detect adrenaline and Pohto and co-workers were

unable to separate adrenaline and dopamine. The present results indicate that the endogenous NA content of rabbit incisor pulp was approximately 50% higher than in feline pulp and 7-8 fold greater than in bovine pulp. In addition, the concentration of DA in the rabbit pulp was only one-fifth of that cited for bovine pulp.

Pre-treatment of rabbits with reserpine or removal of the superior cervical ganglion, reduced the content of NA in the pulpal tissue by approximately 70%, which suggests that most of the NA is contained in storage sites which behave like those in other sympathetically innervated tissues. However, it was noted that much smaller amounts of NA remained in atria after reserpine pre-treatment and in ear arteries after ganglionectomy relative to the amount in pulps, which might imply that some of the NA in dental pulps is stored in non-neuronal sites.

The comparative tissues used, namely atria and ear artery, indicate that reserpine in the former tissue and sympathectomy in the latter respectively greatly reduced the amounts of NA, A and DA remaining in these tissues, as expected. These findings confirm that the two experimental procedures were satisfactory although it should be noted that in earlier experiments not reported, if reserpine was injected into the animal less than 16 hours prior to death, the reduction of catecholamines in pulp and gingiva was much less than if a longer time interval was allowed to elapse, suggesting that reserpine induced depletion of catecholamines in these dental tissues requires 16 hours or longer to be effective. Since no attempt was made to ascertain the time interval after which maximum depletion of any of the three

catecholamines occurred, the lesser effect of reserpine on the level of NA in dental pulp may possibly reflect a time related differential response in different tissues.

Thus the present findings support the contention that NA is likely to be the principal transmitter in the sympathetic system in dental pulp and that most of the amine is stored in sites which respond to sympathectomy and reserpine in the predicted manner.

Verco (1981) studied the effect of unilateral removal of the superior cervical sympathetic ganglion on the content of NA in palatal mucosa and gingiva in rats. He used a fluorescence technique similar to that described by Falck (1962) to demonstrate that denervation was associated with loss of fluorescence on the same side of the animal as the ganglionectomy.

In other studies, Frewin et al. (1971) and Waterson et al. (1974) demonstrated the presence of noradrenergic fluorescence in human gingival tissues, but there do not appear to be reports in the literature quantifying the levels of NA in gingival tissues. The results of this study indicate that the levels of DA and A are much less than NA and are of the same order as those in dental pulp. The greatest amounts of NA were found in gingival tissue from the palate and least in the mandibular buccal gingiva. It is suggested that the levels of NA reported might reflect the nature of the vasculature in the four regions studied, since the anatomical origins of blood vessels and nerves are the same for all four sites. Thus differences might simply be those related to size, with the much thicker palatal gingiva containing a greater number of larger arterioles. It is

intended to examine this possibility later by visualization of the four different gingival sites utilizing fluorescence histochemistry. It is also possible that masticatory function may result in more rapid abrasion and turnover of epithelial cells on palatal and lingual gingiva, and that this more rapid replacement requires greater metabolic activity and therefore a more plentiful and/or responsive blood supply.

Most of the NA in the palatal and lingual gingiva appeared to be stored in reserpine sensitive compartments and could be depleted by removal of the superior cervical sympathetic ganglion; although reserpine pre-treatment and sympathectomy diminished the amounts of NA found in the buccal tissues, the effects were not as marked. The reasons for this are yet to be elucidated. Despite this possible anomaly, the present findings are supportive of the contention that vasomotor control in gingival tissues, like that in dental pulp, is exerted through sympathetic adrenergic nerves (Edwall and Kindlova, 1971).

# CHAPTER 3

# UPTAKE AND METABOLISM OF $^{3}$HNA

# CHAPTER 3

## INTRODUCTION

In the previous chapter catecholamines were shown to be present in gingival tissues, with NA forming the predominant catechol, and therefore the study was extended to examine some characteristics of the uptake and metabolism of NA in these tissues. In the study reported in this chapter, some aspects of the uptake, retention and metabolism of tritiated noradrenaline in excised gingival tissue are reported. As little is known about the sympathetic innervation of gingiva, REA was again included as a comparative tissue for the reasons outlined in the introduction to Chapter 2.

## METHODS

### Uptake of Exogenous NA

Semi-lop-eared rabbits were stunned and bled, after which pieces of gingival tissue were removed as described in the previous chapter, from four sites on each side of the mouth, namely, palate, maxillary and mandibular buccal, and lingual. Two pieces of palatal mucosa were excised for use as control tissues and the central artery (REA) in each ear was exposed, cleaned of surrounding tissue and removed. All sections of gingival tissue and both ear arteries were divided in half, resulting in 16 pieces of gingiva, 4 segments of artery and 2 pieces of palatal mucosa.

Tissues were weighed and equilibrated in warmed, gassed Krebs' solution in four groups containing one segment from each of the five different tissue sources (viz, palatal, maxillary and mandibular buccal and lingual gingiva, as well as REA). The control tissues

(palatal mucosa) were equilibrated separately. For all experiments reported in this paper Krebs' solution contained ethylenediaminetetraacetic acid (0.01 mM) and ascorbic acid (0.3 mM), and was maintained at 37°C and gassed with 95% $O_2$: 5% $CO_2$.

After 60 min each of the four groups of tissues was incubated in 2 ml of Krebs' solution containing tritiated noradrenaline ($^3$HNA) at a concentration of 0.18 uM [0.06 uM(-)$^3$HNA + 0.12 uM(-)NA for periods of 10, 30, 60 and 90 min respectively, after which they were washed in $^3$HNA free Krebs' solution for 40 min. Tissues were blotted on damp filter paper, chopped, and placed in 0.5 ml $HClO_4$ (containing 3 mM EGTA + 0.5 mM $MgCl_2$) at 4°C for a minimum of 16 hr. Subsequently, the level of $^3$H in all samples was determined by liquid scintillation spectrometry. Control tissues were treated similarly, except that the incubation medium did not contain $^3$HNA.

In another experiment, tissues from four animals were processed as described above, but the four groups of tissues were incubated in varying concentrations of $^3$HNA (0.2, 0.6, 0.18 and 3.6 uM respectively) for a period of 60 min. The two control tissues were subjected to identical procedures, but in the absence of $^3$HNA.

Effect of Cocaine on Uptake and Metabolism of Exogenous NA

Segments of gingiva and ear artery were excised as described previously, weighed, cut into 1 mm cubes and equilibrated for 1 hr in Krebs' solution. For these experiments maxillary and mandibular buccal gingiva were pooled. During the last 20 min of the equilibration period, tissues from one side of the animal were placed

in Krebs' solution containing cocaine (30uM) and then incubated in 1 ml of Krebs' solution containing $^{3}$HNA (0.18 uM; viz, 0.06 uM (-) $^{3}$HNA + 0.12 uM (-) NA) and cocaine (30 uM). Tissues from the other side of the animal were placed in Krebs' solution containing $^{3}$HNA only. After 30 min the incubation medium was aspirated and the tissues were washed in two changes of Krebs' solution or Krebs' solution containing cocaine, for 40 min. $^{3}$HNA and its metabolites were extracted from these tissues in 0.5 ml of $HClO_4$ as described previously. The $^{3}$H contents of the incubating media and tissue extracts were determined by liquid scintillation spectrometry. Separation of $^{3}$HNA and its $^{3}$H metabolites from the incubating media and tissue extracts was performed using the cascade column chromatograhic method described below.

Effects of Nialamide on the Metabolism of Exogenous NA

In a further series of experiments, the effects of nialamide on the metabolism of $^{3}$HNA were determined as described for cocaine above except that:

a. nialamide (340 uM) was added to the Krebs' solution after 30 min, and the equilibration period was increased to 80 min,

b. at the end of the incubation period, the tissues were rinsed rapidly in Krebs' solution prior to extraction of $^{3}$HNA and its metabolites.

Purity of $^{3}$HNA

The $^{3}$HNA used in this study was supplied by New England Nuclear as (-)2,5,6 $^{3}$HNA with stated mass and specific activity varying between batches but being of the order of 18 uM and 50 Ci.mmol$^{-1}$. However, it

was found that with all batches used, both mass and specific activity were considerably less than that stated by the manufacturer. Consequently, for each new batch of $^{3}$HNA used in this series of experiments, the mass was assayed, either fluorometrically or using high performance liquid chromatography, and the specific activity determined following liquid scintillation spectometry.

A number of batches was also found to contain unacceptably high levels of tritiated impurities which co-chromatographed with the metabolites of NA, significant in these series of experiments because of the low concentrations of metabolites produced by the dental tissues being investigated. Accordingly, the $^{3}$HNA was purified by adsorption onto alumina, extraction with acetic acid, (0.3 M) freeze-drying and re-constitution in 0.2N acetic acid: ethanol (9:1). The mass was assayed as described above and the specific activity was calculated for each experiment by counting 100 ul of the incubating medium, which had not been in contact with tissues, using liquid scintillation spectrometry. The degradations per minute (DPM) so obtained were related to the known concentration of NA in the samples.

Assay of $^{3}$HNA and $^{3}$H Metabolites

Unchanged $^{3}$HNA and its $^{3}$H metabolites were separated using the cascade column chromatographic method described by Graefe et al. (1973) and summarized in Fig. 2. The catechols were separated initially by adsorption onto alumina. The phenolic metabolites which were not adsorbed were separated on Dowex 50 into fractions containing MOPEG and VMA (fraction 1 termed OMDA) and NMN (fraction 2). DOPEG (fraction 3) and NA (fraction 4) were eluted from the alumina by

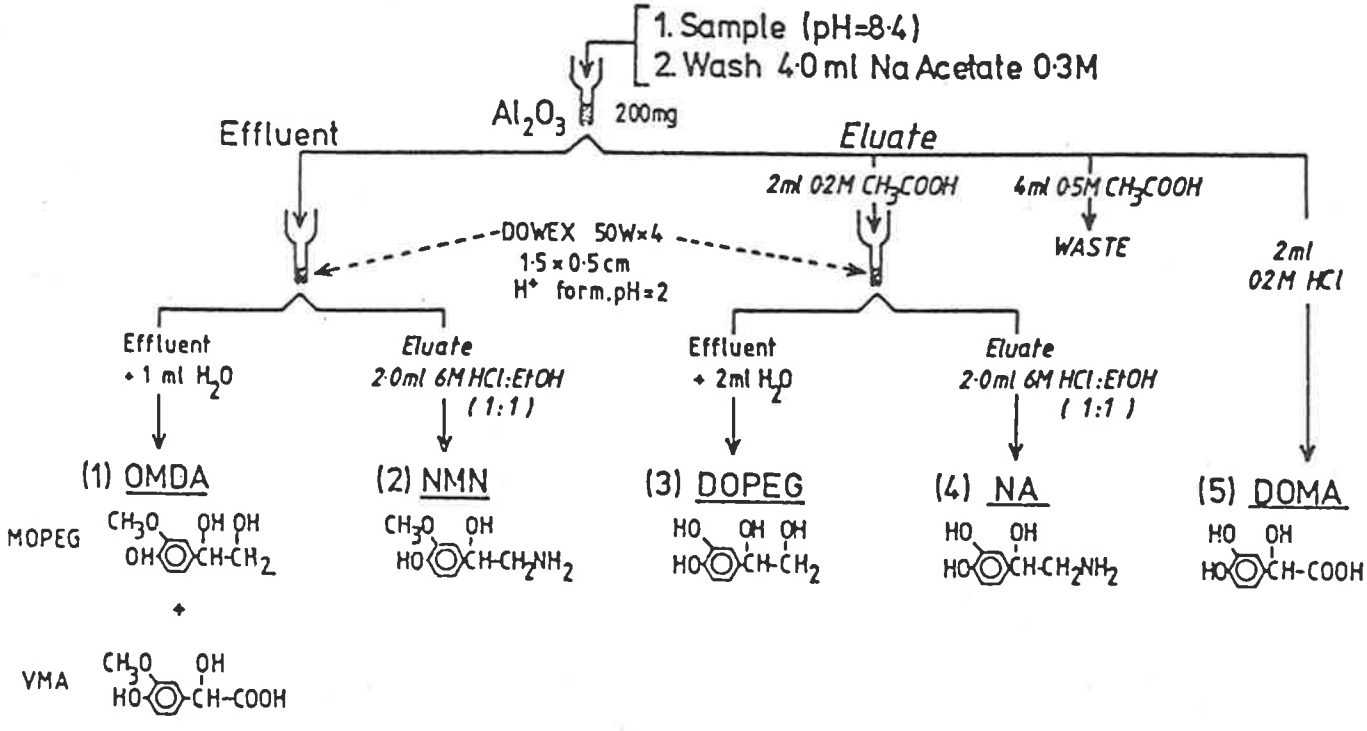


FIGURE 2 A flow diagram of the cascade column chromatographic assay for separating NA and its metabolites. The sample, either 0.8 ml of acidified Krebs solution (incubating medium) or 0.3 ml perchloric acid (tissue extract), were added to 0.1 ml EDTA (0.3 M), 0.1 ml $Na_2SO_3$ (1.0 M), 0.01 ml ascorbic acid (0.6 M), 0.1 ml carrier solution (each $^2$(0.6 M) and 1.0 ml (medium) or 1.5 ml (tissue) of TRIS buffer (1 M, pH = 8.4), before loading the alumina column. Note that MOPEG and VMA both appear in fraction (1).

acetic acid and separated on Dowex 50, whereas DOMA (fraction 5) was eluted subsequently by hydrochloric acid. The level of $^{3}H$ in all fractions was determined by liquid scintillation spectrometry.

The separation and recovery of the metabolites of NA on these columns was measured by native fluorescence after subjecting un-labelled NA and each of the metabolites to the chromatographic procedure described above. The recovery of NA and its crossover into the metabolite fractions were calculated in each experiment following the separation of samples of the $^{3}HNA$ incubating medium which had not come into contact with tissues.

Chemicals and Drugs

(-)2,5,6-$^{3}H$ norepinephrine (New England Nuclear)

(-)noradrenaline bitartrate (Sigma)

Cocaine hydrochloride (McFarlane-Smith)

Nialamide (Pfizer)

RESULTS

Uptake of Exogenous $^{3}HNA$

The amounts of $^{3}H$ retained in the gingival tissues and rabbit ear artery segments after incubation in $^{3}HNA$ (0.18 uM) for 10, 30, 60 and 90 min are indicated in Fig 3. For all tissues, the rate of uptake of $^{3}H$ was greatest during the first 10 min and, generally, was least during the last 30 min; for three of the four gingival tissues (namely, maxillary and mandibular buccal and lingual tissues) there were no significant differences between the amounts of $^{3}H$ retained after 90 min incubation compared with those at either 30 or 60 min

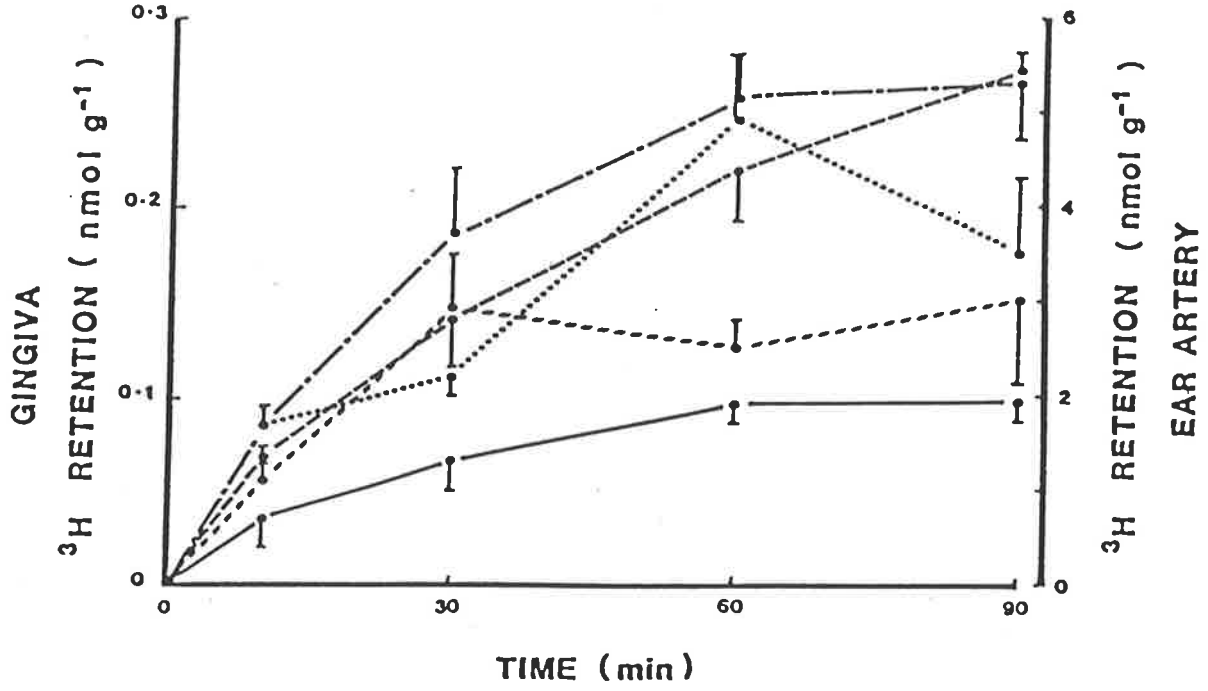


FIGURE 3 Retention of $^3$H in palatal (---), lingual (·····) and maxillary (-·-·-) and mandibular (——) buccal gingiva, and REA (-·——) after various periods of incubation in $^3$HNA (0.18 uM) and followed by washing in $^3$HNA-free Krebs' solution for 40 min. n = 4

Vertical bars represent + or - SEM

(paired t-test; $P > 0.05$). However, the amounts of $^{3}H$ taken up by the ear artery segments and palatal gingiva were significantly greater after 90 min compared with 30 min incubation ($P < 0.05$) but not significant when compared to 60 min incubation ($P > 0.05$). The amount of $^{3}H$ retained by the REA was approximately 20-fold that in palate for all times tested, and in the order of 50-times that in mandibular buccal gingiva. Of the gingival tissues, palate retained the greatest amounts of $^{3}H$ and mandibular buccal gingiva the least; tests of significance revealed that these differences were significant ($P < 0.05$).

The effects of incubating the various tissues in $^{3}HNA$ at different concentrations for one hour are shown in Fig 4. For all tissues, the amounts of $^{3}H$ retained increased with increasing concentrations, so that for REA the amounts retained after incubation in 0.6 and 3.6 uM $^{3}HNA$ were 3 and 11-fold greater than after incubation in 0.18 uM. For palatal and lingual tissues these figures were 2 and 7-fold and for mandibular buccal, 1.4 and 5-fold.

Effects of Cocaine on Uptake and Metabolism of Exogenous NA

Pretreatment of REA and gingival tissues from five animals with cocaine (30 uM) prior to incubation in $^{3}HNA$ (0.18 uM) for 60 min, reduced the level of $^{3}H$ retained by these tissues after 40 min of washing in Krebs' solution compared with untreated tissues (REA - 93%; palate - 82%; buccal - 61%; lingual - 80%).

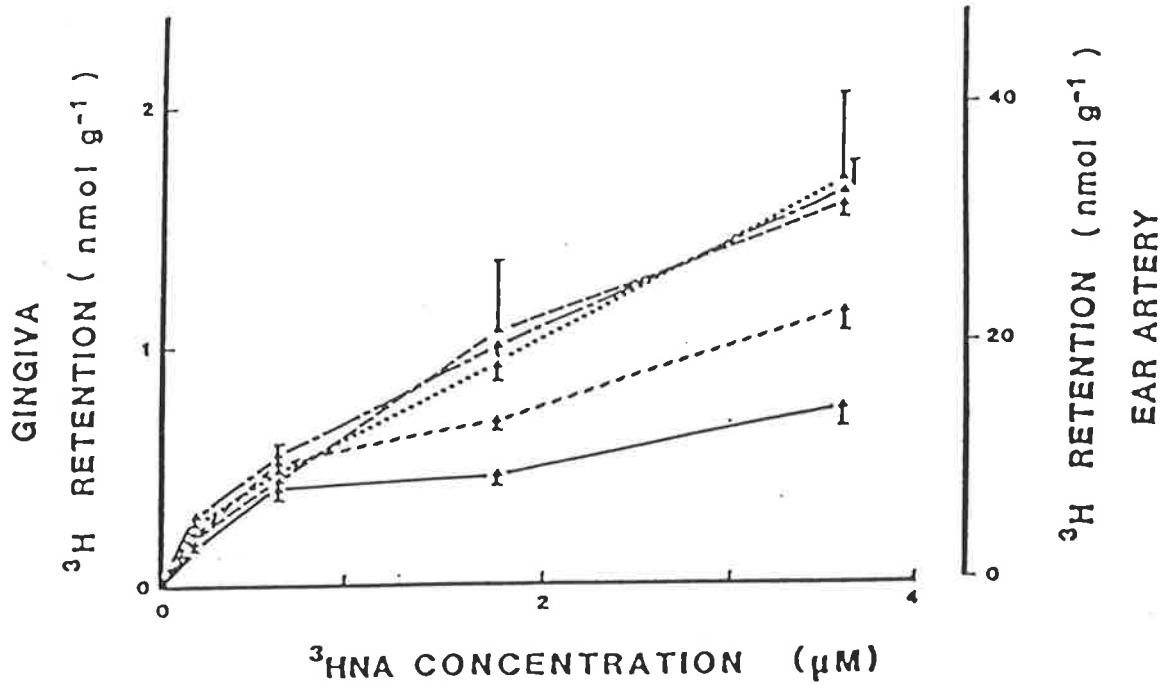


FIGURE 4 Retention of $^3$H in palatal (---), lingual (·····) and maxillary (-·--) and mandibular (——) buccal gingiva and REA (-·-·-) after incubation for 60 min. in $^3$HNA at concentrations shown, followed by washing in $^3$HNA-free Krebs' solution for 40 min. n = 4
Vertical bars represent + or - SEM.

As shown in Fig. 5, the $^{3}H$ which remained in the three untreated gingival tissues was predominantly $^{3}HNA$ (86% in palatal and lingual and 75% in buccal), although small amounts of all metabolites were detected as well. Cocaine significantly reduced the amounts of $^{3}HNA$, $^{3}HDOPEG$ and $^{3}HDOMA$ retained in palate by 89%, 96% and 78% respectively, in lingual tissue by 81%, 89% and 65%, and in buccal by 66%, 62% and 42%. With the exception of the $^{3}HOMDA$ fraction in lingual gingiva, cocaine failed to alter the amounts of the two other metabolite fractions in any of the gingival tissues. In untreated REA segments, almost all the $^{3}H$ comprised $^{3}HNA$ (99%); small amounts of $^{3}HNMN$, $^{3}HDOPEG$ and $^{3}HDOMA$, but not $^{3}HOMDA$, were detected. Cocaine significantly decreased the levels of $^{3}HNA$ and $^{3}HDOPEG$ in these tissues by 94% and 78% respectively, increased the amount of $^{3}HNMN$ 2.3-fold and $^{3}HOMDA$ was increased to detectable levels.

The amounts of the various metabolites assayed in the incubation media following the exposure of untreated and cocaine-treated tissues to $^{3}HNA$ (0.18 uM) are indicated in Fig. 6. There were no significant differences between the amounts of $^{3}HOMDA$, $^{3}HNMN$, $^{3}HDOPEG$ and $^{3}HDOMA$ for each of the untreated gingival tissues, except that in palatal and buccal tissues $^{3}HDOMA$ was formed in significantly greater amounts than $^{3}HNMN$, and in buccal gingiva $^{3}HDOMA$ was also significantly greater than $^{3}HDOPEG$. Cocaine reduced the formation of $^{3}HDOPEG$ in all three tissues and $^{3}HNMN$ in all but palate. By contrast, $^{3}HDOPEG$ was the major metabolite and $^{3}HDOMA$ the smallest present in the incubation media from REA segments. Cocaine almost entirely blocked the formation of $^{3}HDOPEG$ and significantly increased the amount of $^{3}HNMN$ generated.

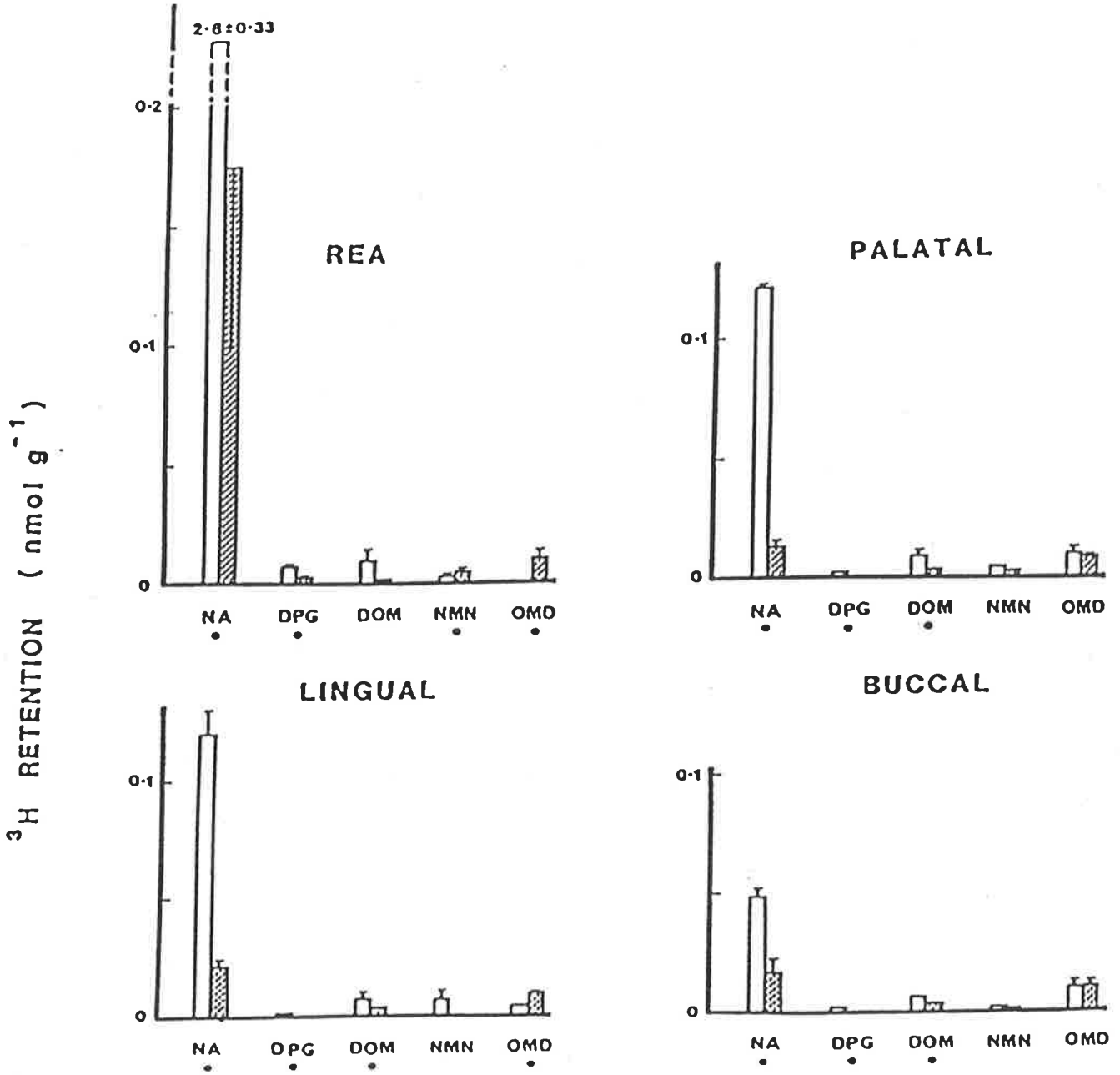


FIGURE 5 The effects of cocaine (30 uM) (hatched columns) on the retention of $^3$HNA and its $^3$H metabolites in palatal, lingual and buccal gingiva and ear artery (REA) following incubation in $^3$HNA (0.18 uM) for 30 min. followed by washing for 40 min. Vertical bars represent + or - SEM. . indicates that the effect of cocaine was significant ($P<0.05$). n = 5.

Abbreviations: NA ($^3$HNA; DPG ($^3$HDOPEG); DOM ($^3$HDOMA); NMN ($^3$HNMN); OMD ($^3$HMOPEG plus $^3$HVMA).

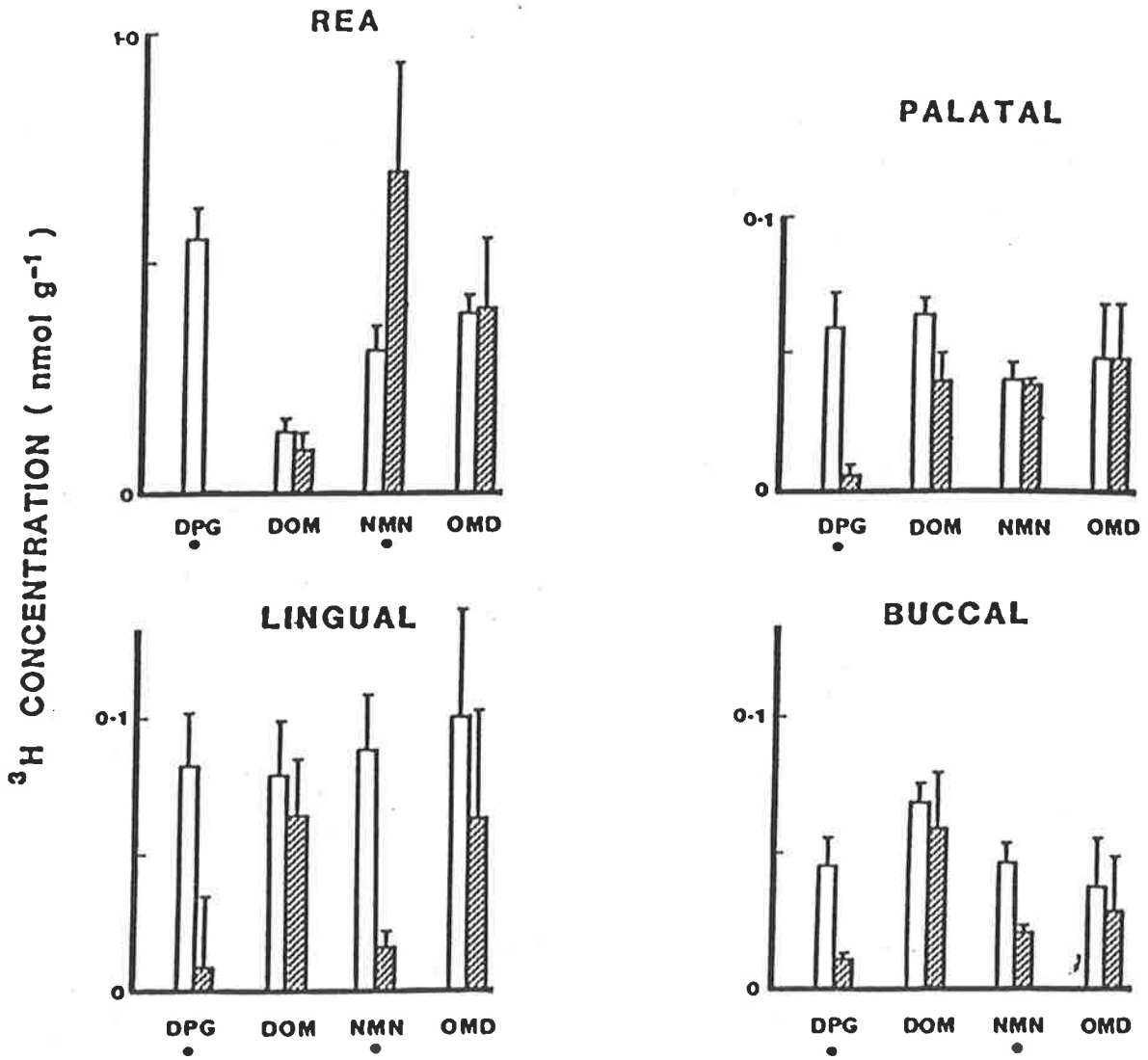


FIGURE 6 The effects of cocaine (30 uM) (hatched columns) on the accumulation of the $^3$H metabolites of $^3$HNA in the incubate media following incubation of palatal, lingual and buccal gingiva and ear artery segments (REA) in $^3$HNA (0.18 uM) for 30 min. Vertical bars represent + or - SEM.

. indicates that the effect of cocaine was significant ($P<0.05$). n = 5.

For abbreviations see legend to Figure 5.

Effects of Nialamide on Uptake and Metabolism of Exogenous NA

Nialamide (340 uM) slightly increased the retention of $^{3}H$ in gingival tissues which had been exposed to $^{3}HNA$ (0.18 uM) for 30 min, although this increase was only significant for buccal tissue.

As shown in Fig. 7, $^{3}HNA$ comprised the largest fraction of the $^{3}H$ retained in the untreated gingival tissues (between 62% and 74%), $^{3}HOMDA$ varied between 10% and 21% and lesser amounts of $^{3}HNMN$, $^{3}HDOPEG$ and $^{3}HDOMA$ were assayed. In all three tissues nialamide significantly reduced the amount of $^{3}HDOPEG$ and $^{3}HDOMA$ retained, but did not alter the levels of $^{3}HNA$, $^{3}HNMN$ and $^{3}HOMDA$. In the REA, 93% of the $^{3}H$ retained in untreated segments was in the form of unchanged $^{3}HNA$, and while treatment with nialamide reduced the amounts of $^{3}HDOPEG$ and $^{3}HDOMA$, the level of $^{3}HNMN$ increased by 80%.

The amount of each metabolite generated during incubation of the tissues in $^{3}HNA$ was calculated by combining the amount in each tissue (Fig. 7) with that in the corresponding incubate (Fig. 8). The results are presented in Fig. 9. It should be noted that there were few differences between the amounts of metabolites formed in any one tissue, except that for palatal and buccal gingiva the levels of $^{3}HDOPEG$ tended to be lower; in palate, $^{3}HNMN$ was present in significantly greater amount than $^{3}HDOPEG$ (P  0.05), while in buccal gingiva, $^{3}HDOMA$ significantly exceeded $^{3}HDOPEG$ (P  0.05).

Nialamide significantly reduced the amounts of $^{3}HDOMA$ and $^{3}HOMDA$ formed in all gingival tissues and almost abolished the formation of $^{3}HDOPEG$ (P  0.05). $^{3}HNMN$ formation was unaltered.

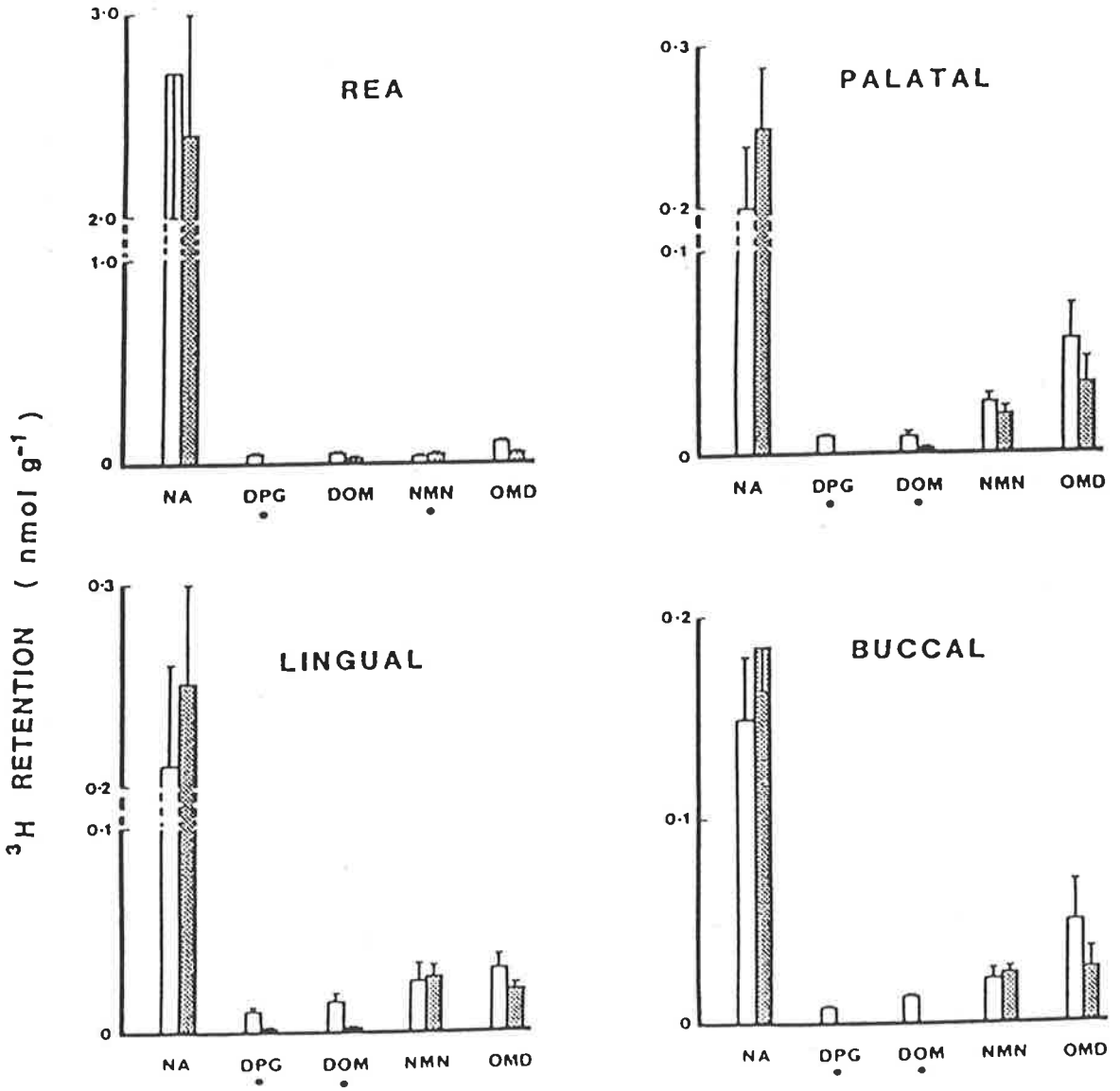


FIGURE 7 The effects of nialamide (340 uM) (hatched columns) on the retention of $^3$HNA and its $^3$H metabolites in palatal, lingual and buccal gingiva and ear artery segments (REA) following incubation in $^3$HNA (0.18uM) for 30 min. and a rapid rinse in Krebs' solution. Vertical bars represent + or - SEM. . indicates that the effect of nialamide was significant ($P<0.05$). n = 5. For abbreviations see legend to Figure 5.

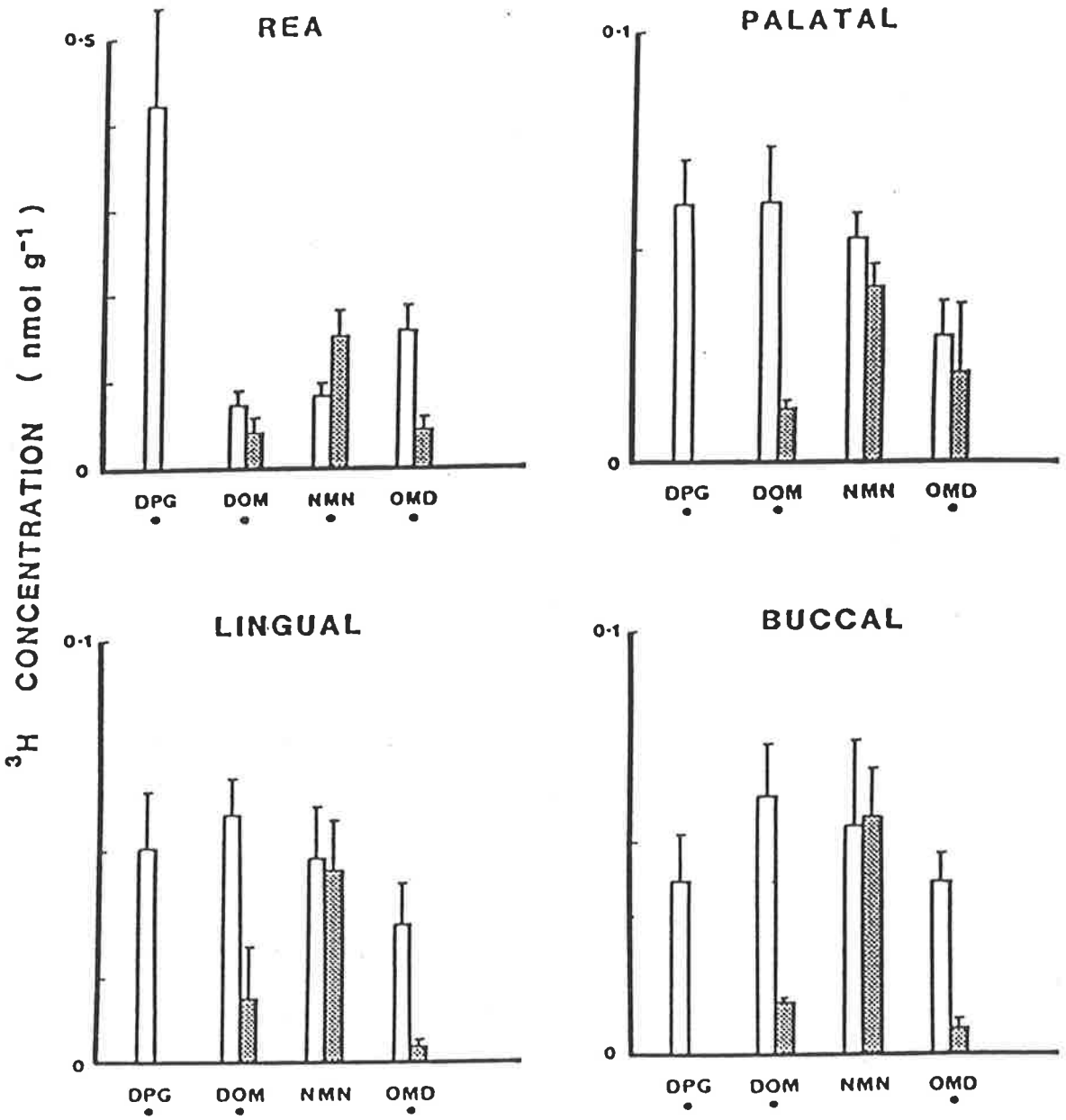


FIGURE 8 The effects of nialamide (340 uM) (hatched columns) on the accumulation of the metabolites of $^3$HNA in the incubate media following incubation of palatal, lingual and buccal gingiva and ear artery segments (REA) in $^3$HNA (0.18 uM) for 30 min. Vertical bars represent + or - SEM. n = 5.

. indicates that the effect of nialamide was significant ($P<0.05$).

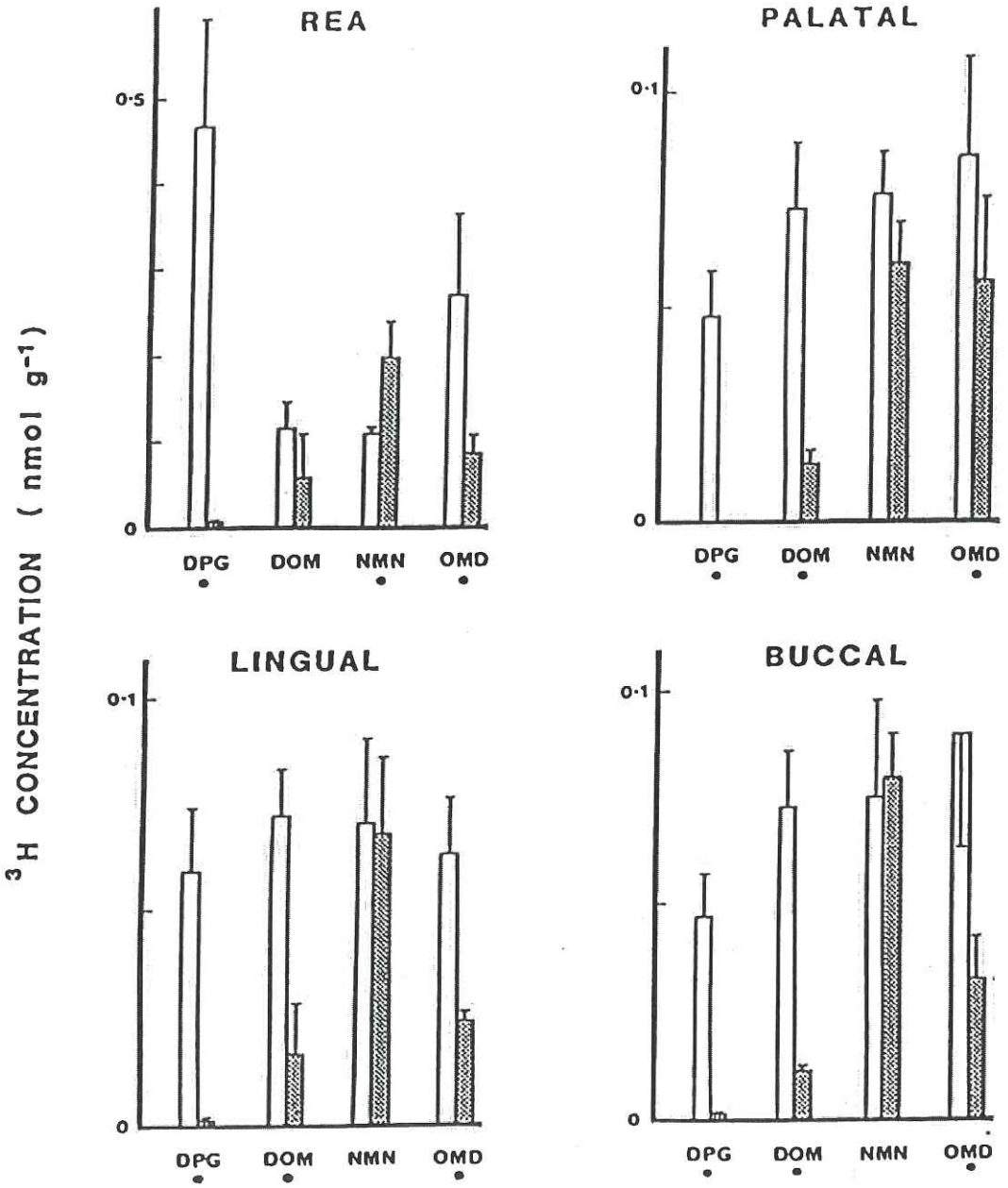


FIGURE 9 The effects of nialamide (340 uM) (hatched columns) on the formation of $^3$H metabolites in palatal, lingual and buccal gingiva and ear artery segments (REA) during incubation in $^3$HNA (0.1 uM). The amount of each $^3$H metabolite formed was calculated by summing the amounts of metabolite in the tissues with the amount which accumulated in the incubation medium. Vertical bars represent + or - SEM. n = 5.

. indicates that the effect of nialamide was significant ($P<0.05$).

For abbreviations see legend to figure 5

In REA segments, the major metabolite generated was $^{3}$HDOPEG. Smaller amounts of $^{3}$HOMDA, and significantly smaller amounts of $^{3}$HNMN and $^{3}$HDOMA were produced. Nialamide significantly reduced the formation of $^{3}$HDOPEG (99%) and $^{3}$HOMDA (68%) and tended to depress the formation of $^{3}$HDOMA (50%), while significantly increasing the amount of $^{3}$HNMN generated by 82%.

## DISCUSSION

The purpose of this part of the study was to investigate some of the properties of sympathetic nerves in gingiva, using REA, a small muscular artery, as a comparative tissue.

As outlined in Fig. 1, monoamine oxidase (MAO) and catechol-O-methyl transferase (COMT) are the enzymes responsible for the metabolism of NA in sympathetically innervated tissues. DOMA and VMA, the acid metabolites of NA and NMN respectively, are produced through oxidation by aldehyde oxidase and DOPEG and MOPEG, the glycol metabolites, through reduction by aldehyde reductase. In most vascular tissues, MAO is the principal intraneuronal enzyme while COMT is the main extraneuronal enzyme. Further, it is recognised that the metabolism of exogenous NA occurs mainly within the nerves by its conversion to DOPEG. However, when neuronal uptake of the catecholamine is inhibited, the metabolic pathway can be diverted through COMT to NMN (Head et al., 1975).

### Uptake of Exogenous NA

The uptake of $^{3}$H into the four gingival tissues following their incubation in $^{3}$HNA (0.18 uM) for periods ranging from 10 to 90 min

indicated that the most rapid accumulation occurred during the first 10 min period and was least for the last 30 min, when the uptake process appeared to be saturated. That the process was not saturated was demonstrated by incubating tissues for 60 min in $^{3}$HNA at concentrations up to 3.6 uM, when the uptake increased between 5 and 7-fold relative to the uptake following incubation at the lower concentration of 0.18 uM. The biphasic nature of the uptake of $^{3}$H into gingival tissue might suggest the presence of a second uptake process responsible for the increased retention of $^{3}$H after apparent saturation of the first. The results also highlighted the greater capacity of the palatal and lingual gingiva to retain $^{3}$H relative to the mandibular and maxillary buccal gingiva.

In comparison, the capacity of the uptake system in REA was substantially greater than that in the gingival tissues at all concentrations of NA used and had not reached equilibrium after 60 min at the lowest concentration. The ability of cocaine to reduce the retention of $^{3}$H in REA by more than 90% is in accord with earlier evidence that it inhibited 90% of the uptake of $^{3}$H following incubation of the same tissue in $^{3}$HNA (0.12 uM) (Head et al. 1975). These results are also in agreement with the concept that most of the exogenous NA retained in tissues following incubation is firmly bound in neuronal stores. Further evidence to support this was obtained from those experiments in which the effects of cocaine on the retention of $^{3}$HNA and its various metabolites were examined. 99% of the total $^{3}$H retained by the untreated REA segments was in the form of unchanged $^{3}$HNA and 94% of this was situated in a cocaine-sensitive compartment.

The findings of the present study in relation to the uptake of $^{3}H$ and $^{3}HNA$ in gingiva and the effects of cocaine on this uptake can be explained in terms of the density of the sympathetic innervation in the tissues as reflected by the endogenous levels of NA in each of them (see Chapter 2). These results showed that REA had the highest content, followed by palate, then lingual gingiva, with buccal gingiva having the lowest content; the capacities of the tissues to retain $^{3}HNA$ after 40 min washing are in the same descending order viz. REA, palatal, lingual and buccal gingiva.

However the finding that only two-thirds of the $^{3}HNA$ which was taken up by the buccal gingiva was sensitive to cocaine might indicate that an extraneuronal compartment exists to which NA was bound sufficiently firmly to resist a long period of washing.

Metabolism of Exogenous NA

The present study confirmed previous findings that DOPEG is the principal metabolite formed during incubation of REA segments in NA (Head et al.1975). The amounts of DOPEG formed in the presence of nialamide, which inhibits deamination by MAO, or of cocaine, which inhibits the uptake of NA into the nerve terminals, were greatly reduced and were accompanied by increases in the amounts of NMN produced. These results imply that whilst metabolism of NA in REA is normally effected by intraneuronal MAO, when this enzyme is inhibited or access of the amine to it is restricted, metabolism is diverted through O-methylation to NMN. The results also support the contention that COMT is situated in extraneuronal sites in this artery.

By comparison, in the gingival tissues, the amounts of DOPEG produced were smaller and the proportions of each of the metabolite fractions were approximately equal. Thus it appears that, unlike REA, metabolism of NA does not have a preferred neuronal pathway. Further, while inhibition of MAO reduced the amounts of DOPEG and DOMA formed, there was no concomitant increase in the amount of NMN produced and hence under the conditions of these experiments it appears that, while both MAO and COMT are implicated in the metabolism of NA, inhibition of MAO does not cause the metabolic pathway to be diverted through O-methylation. The simplest explanation of the apparently diminished role of the sympathetic nerves in the metabolism of NA in the gingival tissue is that less NA is removed by nerves and hence more is available to extraneuronal metabolizing systems. The latter may include the smooth muscle cells of the vessels, and also fibroblasts, since these are present in large numbers in gingival tissue and, in other tissues, are known to contain MAO and COMT (Jacobowitz, 1972; Hume and Waterson, 1978; Azvedo et al., 1980). The effects of cocaine on the metabolism of NA in gingival tissues, namely to inhibit the formation of DOPEG without altering the production of DOMA, suggest that while the former is produced intraneuronally the latter is produced extraneuronally. Further, as the amounts of NMN detected in two of the three gingival tissues were decreased by cocaine it is possible that COMT is situated intraneuronally. The latter implication is surprising, since in the majority of tissues COMT is not present in the nerves (Trendelenberg, 1981). Furthermore, there is no evidence of neuronal COMT in the REA (Head et al, 1977), although the sympathetic nerves to this vessel originate from the same ganglion as that supplying gingival tissue.

## CONCLUSION

These investigations into the catecholamine content and adrenergic mechanisms of rabbit gingival tissue produced some surprising results. The initial assays of endogenous catecholamine contents were carried out on gingival tissues from four different sites, with little expectation of differences since the neuronal and vascular supplies to both maxillary and mandibular oral tissues originate from the same sources. The catecholamine content of both maxillary and mandibular tooth pulps was similar, but that of the several gingival tissues was unexpectedly and significantly different; this difference was also reflected in the patterns of uptake and metabolism of exogenous NA by the gingiva. Palatal tissues, with the highest endogenous content, demonstrated the greatest capacity for uptake and most closely resembled the REA in its metabolic activity, while buccal gingiva, with the lowest endogenous levels and least uptake capacity, also demonstrated the most contrasting metabolic pattern. The differences noted might be explained by differences in the size and distribution of the vasculature in the sections of tissue removed. Thus gingiva with larger or more arteries/arterioles might display characteristics more closely resembling the isolated artery than other gingival tissues with fewer and smaller vessels.

The cocaine-sensitive O-methylation of NA noted raises a series of interesting questions. For example, is COMT present intraneuronally which would be unusual in vascular tissues, or is it in a non-neuronal compartment? In some preliminary experiments not reported here, unilateral removal of the superior cervical ganglion did not result in diminution of the O-methylation of $^{3}$HNA compared with the unoperated

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side. These results suggest that COMT is situated in a cocaine-sensitive extraneuronal site within the tissue. Other results from this laboratory have demonstrated an O-methylating cocaine-sensitive extraneuronal compartment in dental pulp, which might be associated with fibroblasts (Parker, D.A.S. personal communication). Further experimentation is required to elucidate these and other interesting questions.

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