THE UPTAKE AND METABOLISM OF NORADRENALINE BY THE RABBIT UTERUS

A thesis submitted for the degree of Doctor of Philosophy

by Jennifer Ann Kennedy, Department of

Human Physiology and Pharmacology,

University of Adelaide,

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SUMMARY

1) The aim of this study was to examine whether the uptake and metabolism of noradrenaline (NA) in the rabbit uterus was altered by 17β-oestradiol and progesterone, and by pregnancy.

2) Earlier evidence, in the rat and human, indicated that monoamine oxidase (MAO) activity was increased by conditions of progesterone dominance and decreased by conditions of oestrogen dominance. Pregnancy did not alter the MAO activity of the rat uterus. Changes in catechol-o-methyltransferase (COMT) activity associated with different stages of the oestrous cycle were equivocal, but a two fold increase in activity had been reported to occur in the rat uterus during pregnancy. The only data available on the metabolism of catecholamines in the intact uterus indicated that adrenaline (A) was o-methylated to a greater extent during the oestrous phase of the cycle. Data on the rabbit was confined to reports that the endogenous noradrenaline content was increased by 17β-oestradiol, and decreased by progesterone or during late pregnancy.

3) In the present study it was shown that the endogenous NA concentration of the uterus was inversely related to the uterine weight in ovariectomized animals. The concentration of endogenous NA was found to decrease with 17β oestradiol, 17β-oestradiol + progesterone treatment and pregnancy but not with progesterone treatment alone. Since the NA content of the whole uterus was unaffected by any of the treatments, the changes in concentration of NA were interpreted as a consequence of changes in uterine weight.

4) 17β-oestradiol treatment increased the MAO and COMT activity of the rabbit uterus, as tested in homogenates, whereas progesterone increased only the MAO activity. Pregnancy caused a small increase in MAO activity. These changes were different from those reported earlier in the rat and human.

5) The major metabolites of ^{3}H1-NA generated by rabbit uterine segments *in vitro* were 3,4, dihydroxyphenyl glycol (DOPEG; 40%) normetanephrine (NMN; 19%) and 3, methoxy 4, hydroxy phenyl glycol (MOPEG; 29%). The effects of cocaine on metabolite formation indicated that DOPEG was largely neuronal in origin, and the o-methylated metabolites were largely extraneuronal in origin. A positive correlation between the endogenous NA concentration and ^{3}H DOPEG formation in the rabbit uterus represented further evidence of the neuronal origin of this metabolite.

6) The most striking change in metabolite distribution was an increase in NMN formation produced by progesterone treatment and by pregnancy. The effects of 17β-oestradiol were equivocal in that it caused a significant increase in DOPEG formation in one series of experiments but not in a second series. However it did not affect NMN formation in either series.

7) Since progesterone treatment and pregnancy did not increase the COMT activity of the uterus (tested in homogenates) the increase in NMN formation was interpreted as evidence that these treatments resulted in increased access of NA to the enzyme. The possibility that the increase in o-methylation reflected a decrease of the neuronal uptake and metabolizing systems seems excluded since cocaine, which inhibits DOPEG formation, does not increase o-methylated metabolite formation.

8) Evidence is presented that the effects of the steroids are specific for the uterus in so far as neither steroid, nor pregnancy, altered the uptake and metabolism of NA in a non-reproductive peripheral tissue (rabbit ear artery).

9) Some less extensive studies on other reproductive tissues indicated that the rabbit oviduct, and ovary, have quite different patterns of metabolite formation. In the oviduct, the distribution is similar to that in the rabbit ear artery in that DOPEG is the major metabolite, pointing to a largely neuronal metabolism of NA. In the ovary, o-methylation predominates to a greater extent than in the uterus. These changes accord with the higher density of innervation (as tested by the endogenous NA concentration) in the oviduct than in the other tissues. Although the effects of steroids on these two tissues were not examined, the possibility that their effects may differ from those on the uterus was suggested by the finding that pregnancy did not affect the formation of any metabolite in the oviduct and was associated with a decrease in the formation of all metabolites in the ovary.

10) As a result of this study, it was concluded that the activities of the NA-metabolizing enzymes are altered by steroid treatment in different ways in different species. Furthermore, even in the one species, the changes do not bear a simple relationship to the metabolism of NA in the intact uterus. The possible functional significance of these changes is discussed, and it is suggested that

the predominance of extraneuronal o-methylation during pregnancy may be part of a protective mechanism to minimize the impact on uterine activity of fluctuations in circulating catecholamines.

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.

JENNIFER ANN KENNEDY

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PUBLICATIONS

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ABBREVIATIONS

A	adrenaline
NA	noradrenaline
DOPEG	3,4 dihydroxyphenylglycol
MOPEG	3, methoxy, 4, hydroxyphenylglycol
VMA	vanillyl mandelic acid
DOMA	3,4, dihydroxy mandelic acid
COMT	catechol-o-methyl transferase
MAO	monoamine oxidase
TLC	thin layer chromatography
D-50	Dowex 50
Al_2O_3	alumina
HCl	hydrochloric acid
HAC	acetic acid
^3H	tritium
NaOH	sodium hydroxide
NMN	normetanephrine

CHAPTER 1

INTRODUCTION

The mammalian uterus is innervated by sympathetic nerves arising in the lumbar region of the spinal cord. There has been disagreement as to whether it receives a parasympathetic innervation, but no convincing evidence has been provided for the presence of parasympathetic nerves except in the uterine arteries of a few species.

The sympathetic innervation of the uterus has been reviewed by a number of authors (Gruber 1933, Marshall 1970, 1973, Pauerstein & Zaunder 1970, Bell 1972) but its function remains obscure. Nevertheless the density of uterine innervation, the response of the effector cells to catecholamines and the activity of the catecholamine metabolizing enzymes of the uterus have all been shown to alter with different steroid hormone states and pregnancy.

It is proposed in this introduction to compare, initially, the hormone states of the rabbit with several other species and then to review the changes in sympathetic innervation which occur with different hormone states. A brief discussion of the suggested functions of the sympathetic innervation of the reproductive tract will be included. The aims of the present study will then be considered in relation to the literature on NA metabolism in the uterus.

1. HORMONE STATES

Unlike the rat, guinea pig and human, the rabbit does not undergo repeated sexual cycles. It ovulates only after mating and is termed an 'induced' or 'reflex' ovulator. Mating causes a reflex release of luteinizing hormone which initiates ovulation. Hilliard & Eaton

(1971) reported that 17β oestradiol and progestagens (mainly 20 α hydroxyprogesterone) secretions from the ovary increase following coitus in the rabbit, reaching a maximum after 90-120 minutes. 17β oestradiol levels increase three fold over those in the oestrous rabbit. During the 3 days of tubal transport of the ova, both oestrogen and progesterone secretion are very low. Following their entry into the uterus, the blastocysts remain in the lumen for 4 days and space evenly along the uterus before implantation. On days 7-8 after mating, there is a transient surge in 17β oestradiol levels prior to implantation which then decline by day 10 and remain relatively constant throughout the rest of the gestation period (Hilliard *et al.*, 1973). Progesterone levels increase following implantation reaching a peak at day 14 to 18 post coitum and remaining high until the last week of pregnancy. At day 30-32 progesterone output drops to low levels but there is no evidence for a decline or surge in 17β oestradiol secretion prior to parturition. Luteal progesterone is necessary for the maintenance of pregnancy in the rabbit for the first 30 days of pregnancy since ovariectomy at any time during these 30 days results in foetal death (see Hilliard *et al.*, 1973).

In contrast to the rabbit, the rat, guinea pig and human undergo repeated oestrous cycles and are termed 'spontaneous' ovulators. Oestrogen and progesterone secretion fluctuates during the oestrous cycle. In the rat there is a proestrous increase in 17β oestradiol secretion (Yoshinager *et al.*, 1969) and in progesterone secretion (Holzbauer & Yondim 1973). Progesterone levels are low at oestrous when 17β oestradiol is elevated but following oestrous, 17β

oestradiol levels decline and progesterone increases so that by metoestrous and dioestrous, progesterone levels are high and 17β oestradiol low. A similar change in steroid secretion has been reported for the guinea pig (Blatchley *et al.*, 1976). In the rat, oestrogen output drops to intermediate values during gestation but rises prior to delivery reaching a maximum on day 21 just prior to delivery (Yoshinager *et al.*, 1969). In the guinea pig and human, oestrogen is derived principally from the placenta or foetoplacental unit and reaches its highest levels of secretion just prior to parturition. In both species progesterone secretion is elevated throughout pregnancy and produced predominantly by the placenta (Bedford *et al.*, 1972).

2. INNERVATION OF THE UTERUS

(a) Rabbit

Following the early studies of Frankenhäuser (1867) on the human uterus, Langley & Anderson (1895 a & b, 1896), using dissection studies and denervation techniques, demonstrated that the rabbit uterus was innervated by discrete hypogastric nerves. These nerves arise in the 3rd to 5th lumbar segments of the spinal cord and communicate via the 4th to 6th lumbar sympathetic ganglia to the inferior mesenteric ganglia. Two hypogastric nerves arise from each inferior mesenteric ganglion and then divide into two branches, the dorsal branch connecting with the pelvic plexus and the ventral branch joining ganglia in the dorsolateral wall of the vagina. Electrical stimulation of the hypogastric nerves caused contraction of the rabbit

uterus and pallor due to constriction of uterine blood vessels. Since the ganglion blocker, nicotine, abolished the contractile response of the uterus but not the vasoconstriction, Langley & Anderson (1895b) concluded that sympathetic fibres in the hypogastric nerves running to the smooth muscle of the rabbit uterus were preganglionic, synapsing in ganglia close to the uterus; those to the blood vessels were postganglionic fibres. Since stimulation of the pelvic nerves had no effect in the uterus they concluced that the uterus lacked a parasympathetic innervation (Langley & Anderson 1895a).

In 1962, Falck described the histochemical method of localizing NA within nerve terminals utilizing the fluorescence developed when catecholamines were condensed with formaldehyde vapour. The use of this technique in conjunction with the development of specific assays for NA and A within tissues (Bertler *et al.*, 1958, Häggendal 1963) enabled a more detailed study of the innervation of the female reproductive tract by a group of Swedish workers (see Sjöberg, 1967). In the rabbit uterus, fluorescent varicose nerve terminal were shown to run in close connection with the smooth muscle cells of the uterus, both in the outer longitudinal and inner circular smooth muscle layers of the myometrium. Similar green fluorescent terminals were found enclosing blood vessels in the vascular plexus between these two smooth muscle layers. No regional variation in the density of innervation has been reported for the rabbit uterus.

Schofield (1952) and Miller & Marshall (1965) queried the preganglionic nature of the fibres in the hypogastric nerve in that

they could not demonstrate blockade of the contractile response of the rabiit uterus to hypogastric nerve stimulation using hexamethonium. However the results of Owman *et al.* (1966) supported the earlier findings of Langley & Anderson in that section of the hypogastric nerves and removal of L_3-S_2 sympathetic ganglia in the rabbit abolished fluorescence in ovarian nerves and uterine perivascular nerves but not in the myometrial smooth muscle innervation. Since section of postganglionic sympathetic nerves leads to the disappearance of fluorescence after 48 hours (Falck 1962) the results of Owman *et al.* (1966) indicated that the postganglionic nerves to the uterine smooth muscle had their origins beyond the point of section i.e. near the uterus.

The rabbit myometrium has been examined with the electron microscope by Hervonen & Kanerva (1972, 1973). It was shown to contain approximately 75% adrenergic terminals and 25% non-adrenergic terminals. These nerves were found mostly in the interstitial spaces between bundles of smooth muscle cells not within the bundles themselves. The terminal varicosities were found close to the smooth muscle cells, but the gap (2000 Å) was larger than that described for other smooth muscle. Both adrenergic and non-adrenergic nerves were seen to be in close contact but the significance of these connections is not known. In view of the lack of cholinesterase staining in the rabbit uterus (Jordan 1970, Owman & Sjöberg 1966) these nerves do not appear to be cholinergic.

The degree of parasympathetic innervation to the uterus is still in dispute. However, Bower (1966) confirmed the findings of Langley &

Anderson in that he was unable to demonstrate action potentials in the parauterine nerves in the rabbit after stimulation of pelvic roots. When combined with the lack of cholinesterase staining, it appears that the rabbit uterus, at least, does not have a parasympathetic nerve supply.

(b) Other Species

Similar arrangements of adrenergic terminals to those in the rabbit uterus have been described for the uteri of the guinea pig, cat and human (Owman & Sjöberg 1966, Owman *et al.*, 1967, Rosengren & Sjöberg 1967). However, the human uterus (Owman *et al.*, 1967) and the guinea pig uterus (Thorbert *et al.*, 1977) display regional variations in the density of innervation, the cervix possessing a more dense innervation than the rest of the uterus. There is also species variability in the overall density of innervation of the uterus, the cat uterus being more densely innervated than that of the guinea pig or rabbit (Sjöberg 1967) while the rat uterus has a sparce innervation, nerves being mainly confined to the vasculature (Norberg & Fredricsson 1966, Falck *et al.*, 1974). Adrenergic ganglion formations were also found in the uterovaginal junction, in the connective tissue layer close to the vagina and in the vaginal wall in the cat, rabbit, guinea pig and human (Sjöberg 1967).

At the ultrastructural level, Silva (1967) found that the innervation of the rat myometrium was similar to that described for other tissues in that the autonomic ground plexus consisted of non myelinated fibres surrounded by Schwann cells ramifying within the myometrium. Single axons were found to be within 200 to 300 Å of the smooth muscle sarcolemma but were not definitely identified as nerve

terminals. A similar arrangement was reported for the guinea pig myometrium (Isaac *et al.*, 1969). The density of innervation was low, particularly in the rat as indicated above. However, as pointed out by Marshall (1970), conduction via tight junctions between smooth muscle cells in the myometrium (Bergman 1968) may reduce the necessity for a dense innervation.

There appears to be a species variability in the degree to which the uterus is innervated by short postganglionic neurons. Issac *et al.* (1969) attributed the lack of decline of catecholamine fluorescence after hypogastric nerve section in the guinea pig uterus to the fact that a substantial amount of its innervation appears to be via the ovarian nerves. As a result of experiments with hexamethonium and EM examination of the ovarian and hypogastric nerves they suggested that the guinea pig uterus was mostly innervated by long postganglionic fibres from the ovarian nerves. Re-examination of the guinea pig uterus by the Swedish workers (Thorberg *et al.*, 1977) confirmed the findings of Issac *et al.* (1969) in that a large proportion of the uterus was innervated by nerves from the ovarian end of the uterus, the cervix being innervated by the hypogastric nerves. The innervation of the rabbit uterus has not been re-examined in the same way. However, since removal of L_3-S_2 ganglia in the rabbit abolished NA fluorescence in the ovarian nerve but not that associated with nerves in the smooth muscle of the myometrium (Owman *et al.*, 1966), it is probable that there is a species difference and the rabbit uterus is innervated via short postganglionic fibres from the hypogastric nerves.

Changes in the Density of Uterine Innervation

(a) Rabbit

Rosengren & Sjöberg (1968) have demonstrated an increase in the NA content of the whole rabbit uterus in early pregnancy which nevertheless corresponds to a decreased concentration of NA and decreased density of innervation due to the greater increase in uterine mass. In late pregnancy the total NA content fell below the level in the non-pregnant uterus corresponding to a very low density of innervation. Total ovarian NA content was unaltered whereas the content of the whole rabbit oviduct increased so that it maintained its NA concentration and density of innervation.

Pretreatment of rabbits with 17β oestradiol (0.5 μg/Kg) for 7 or 14 days increased the NA content of the whole uterus and the intensity of NA fluorescence in uterine nerves (Sjöberg 1968a, Falck *et al.*, 1969a) although the concentration of NA decreased due to the stimulatory effect of oestrogen on uterine growth. However, Miller & Marshall (1965) were unable to detect a significant change in the NA content of the whole rabbit uterus after a higher dose of 17β oestradiol for 4 days although NA concentration decreased significantly. Falck *et al.* (1969b) found that concurrent treatment with progesterone (2 mg/Kg) after oestrogen priming reversed the oestrogen effect and caused a decrease in the NA content of the whole uterus and a large decrease in the density of innervation since the combined steroid treatment further increased the size of the uterus. Miller & Marshall

(1965) demonstrated a significant decrease in the NA concentration but not the NA content of the whole uterus after 17β oestradiol and progesterone treatment.

Falck *et al.* (1969b) suggest that changes in the NA content of the uterus during pregnancy may be the result of changes in the secretion of these two hormones and further that such changes may be a unique property of tissues innervated by short postganglionic fibres since the heart and ovaries did not show similar changes.

At the ultrastructural level, Hervonen *et al.* (1972) using twice the dose of oestrogen to that used by Sjöberg (1967) demonstrated that the adrenergic nerve terminals in the rabbit myometrium contain vesicles with a greatly increased density of granules. Moreover the axons contained increased quantities of neurotubuli.

(b) Other species

In the rat uterus, the NA content is low and unaffected by the hormonal status of the animal, whereas its A content increased during oestrous and after oestrogen administration (Rudzic & Miller 1962, Wurtman *et al.*, 1964, Cha *et al.*, 1965) as well as during pregnancy (Cha *et al.*, 1965). However the rat appears to be unique in that A levels are very low in other species such as the rabbit and guinea pig (Miller & Marshall 1965, Sjöberg 1967). A also appears to be localized at a non-vesicular site in the rat uterus (Wurtman *et al.*, 1964). Wurtman *et al.* (1964) suggested that these changes in the rat uterus were due to increased binding of A by the uterus and increased delivery of A to the uterus since *in vivo* the pregnant and oestrogen-treated

rat uterus bound more ^{3}H A than the dioestrous uterus and Green & Miller (1966c) demonstrated that plasma A increased during pregnancy in the rat. *In vitro*, the uterus from oestrous rats was found to accumulate more ^{3}H A than those from dioestrous rats (Green & Miller 1966a) although the accumulation of ^{3}H A was cocaine sensitive indicating that it was probably neuronal and therefore may not explain the changes in endogenous A.

In the guinea pig, the density of adrenergic innervation has been shown to decline during pregnancy (Sjöberg 1968b). This effect was not related to stretch of the uterus since it also occurred in the uterine horn not containing foetuses in unilaterally pregnant guinea pigs. The NA content of the guinea pig uterus but not of the rat uterus was shown to decrease after ovariectomy and the authors suggested that such an effect may have been due to a fall in oestrogen secretion since it could be counteracted by administration of 17β oestradiol (Falck *et al.*, 1974). The NA contents of the longitudinal muscle of the guinea pig myometrium and of the costo uterine muscle have been shown to decline at dioestrous when progesterone levels are elevated provided guinea pigs of similar body weight are used (Hanberg *et al.*, 1978). In view of the fact that a large proportion of the guinea pig uterus appears to be innervated by long postganglionic fibres (Issac *et al.*, 1969) it seems unlikely that such changes in NA concentration of the uterus can be related specifically to short post ganglionic fibres as suggested by Falck *et al.* (1969b). It seems more likely that the specificity of the effect may be due to the uterus being a target organ with specific receptors for the ovarian steroids

(El-Banna & Sacher 1977). Bell & Malcolm (1978) showed a similar loss of NA fluorescence in perivascular nerves of the guinea pig uterus and gastrocnemius muscle following local implantation of progesterone suggesting that long post ganglionic sympathetic neurons respond in a similar way if the local concentration of steroid is high.

The mechanisms by which oestrogen and progesterone exert their effects on NA content of the uterus has not been established. Possible mechanisms include 1) changes in synthesis 2) changes in vesicle binding 3) changes in release 4) changes in uptake and 5) changes in catabolism of NA.

3. FUNCTIONAL SIGNIFICANCE

The functional significance of the innervation of the uterus and of the female reproductive tract as a whole is still uncertain. Spinal section has been said to have little effect on conception and parturition (Reynolds 1965, Anderson *et al.*, 1963) although the length of labour may be altered by some spinal injuries (Kuntz 1953, Jeffcoate 1959). Nevertheless it is likely that the sympathetic innervation is capable of modifying function in the reproductive organs.

The role of sympathetic nerves in ovulation has been reviewed by Bahr *et al.* (1974). The outer wall of the ovarian follicle has been shown to contain smooth muscle which receives a sympathetic innervation in a number of species including the rabbit and human (Owman & Sjöberg 1966, Owman *et al.*, 1975). Attempts to denervate the

ovary have given conflicting results on ovulation. Weiner *et al.* (1975) failed to show any effect of denervation on subsequent HCG induced ovulation in the rabbit ovary and ovaries transplanted to other sites in the body have been shown to release ova (see Bahr *et al.*, 1974). However, Nalbandov *et al.* (1973) demonstrated that intrafollicular injections of α blockers, NA synthesis inhibitors and storage depletors all blocked ovulation in the rabbit, although the block could be overcome by the injection of an appropriate ratio of FSH and LH.

The sympathetic innervation of the oviduct has been reviewed by Paton *et al.* (1977). The oviduct has a particularly dense innervation in the circular smooth muscle layer of the isthmus but a fairly sparce one in the longitudinal muscle layer (Brundin 1965). It has been suggested that the circular smooth muscle layer of the isthmus acts as a sphincter controlling the rate of ovum transport through the oviduct into the uterus (Pauerstein *et al.*, 1974). Stimulation of the nerves to the oviduct have been shown to cause α mediated contraction in the rabbit oviduct whether oestrogen or progesterone dominated (Kennedy & Marshall 1977) although β receptor activity increases under progesterone dominance (Hodgson & Pauerstein 1974). However Hodgson *et al.* (1975) were unable to alter fertility in the rabbit by administration of α or β blockers or by reserpine treatment at the time of ovum transport. The effects of local infusions of 6-hydroxydopamine were difficult to assess due to the effects of the vehicle. Unfortunately, although the doses of reserpine and 6-hydroxydopamine used in their study had previously

been shown to deplete the oviduct of NA, there is some doubt thrown upon their results in that they did not confirm the loss of NA in the oviducts used in the study cited here.

The supporting muscle layers of the oviduct and uterus also receive a sympathetic innervation e.g. the mesotubarium superius in the rabbit (Doteuchi & Takeda 1978) and costouterine muscle in the guinea pig (Pennefather 1978) and it has been suggested that they might aid in alignment of the ovary and oviduct at the time of ovulation although as yet no evidence has been presented for such a role.

The early studies of Langley and Anderson showed that the virgin rabbit uterus contracted in response to hypogastric nerve stimulation and its blood vessels constricted. Subsequently, Rudolph & Ivy (1930) described the so-called pregnancy reversal phenomenon in the rabbit, the response of the pregnant uterus to nerve stimulation being relaxation. Miller & Marshall (1965) further characterized this phenomenon in the rabbit as being due to increased β receptor activity during pregnancy and showed that it could be produced by administration of progesterone. 17β oestradiol administration caused an increase in α receptor activity mediating uterine contraction in response to nerve stimulation.

The rat on the other hand shows the reverse effect, constricting in the pregnant animal and relaxing in the non-pregnant animal (see Marshall 1970). The human uterus also displays increased β receptor activity during pregnancy (Nakanishi *et al.*, 1968) a fact which has been utilized clinically to delay labour by the use of β adrenoreceptor agonists (Lindmark *et al.*, 1973).

The uterine vasculature is sensitive to α constrictor influences but has little capacity for β mediated dilatation in the rabbit (Graham & Sani 1971) or other species studied (Bell 1972). Unlike the effects on myometrial smooth muscle there is no evidence for the development of a β-mediated vasodilatation under progesterone dominance in the uterine vasculative.

As with the ovary and oviduct, a clear role for the sympathetic innervation of the uterus has not been established. Uterine motility is involved in the transport of sperm from the cervix to the oviduct (Thibault 1973), in the distribution and spacing of blastocysts within the uterus at the time of implantation (Boving 1954) and in expulsion of the foetus at parturition. Whether the sympathetic nerves have a modifying influence in one or all of these functions has not been established. It has been suggested that there is a sympathetically mediated inhibitory tone in the rat uterus during early pregnancy (Deis & Pickford 1964) and in the rabbit (Sabanah *et al.*, 1968) since administration of sympatholytic agents and β blockers increased uterine activity *in vivo*. α blockers caused uterine inertia in the rabbit at term. However the results of Sabanah *et al.* (1968) have been criticized (Bell 1972) since no allowance was made for the effects of these drugs on uterine blood flow or central pathways e.g. those controlling the release of oxytocin. Oxytocin has been suggested as a major factor in rabbit uterine contractility at parturition with the removal of a pre-existing progesterone block (Csapo 1969). However sympathetic activity may have a permissive role in the action of oxytocin since Rüsse & Marshall (1970) have demonstrated that subthreshold stimulation of the hypogatric nerves potentiate the response of the oestrogen-

dominated guinea pig uterus to oxytocin. However the large reduction in density of the sympathetic nerves in the uterus near term suggests a reduced role of the innervation in parturition. The increased β receptor activity of the uterus during pregnancy in a number of animals including the rabbit has also been interpreted as a protective mechanism against the effects of circulating catecholamines (Pauerstein & Zaunder 1970) rather than contributing to a nerve-mediated tonic inhibitory influence. The cat must pose an interesting exception to either of these hypotheses since hypogastric nerve stimulation causes contraction of the pregnant cat uterus. However there is some evidence that its response reverts to relaxation near term (del Pozo, 1946).

In summary, no conclusive evidence has been obtained that the sympathetic nerves to the reproductive organs normally participate in their function. Denervation studies have been inconclusive in that they have not been sufficiently specific. Thus, although 6 hydroxy-dopamine treatment was shown to produce reduced implantation and increased foetal mortality in mice and rats (McDonald & Airaksinen 1974) this type of denervation technique is not specific for the reproductive organs. Reduced fertility may have reflected changes in other systems e.g. reduced systemic blood pressure may have adversely affected perfusion of the reproductive organs.

There is also a need to differentiate the role of the nerves supplying the blood vessels of the reproductive organs from that of the nerves supplying the smooth muscle of the organs themselves.

4. METABOLISM OF NA

Studies on a variety of tissues have established that a major mechanism of inactivating catecholamines in tissues involves their uptake by cells and subsequent metabolism by monoamine oxidase and catechol-o-methyl transferase. It is proposed to summarize the major features of this inactivation process and to describe in more detail studies on the mechanism of inactivation of NA in the female reproductive tract.

(a) Uptake of Catecholamines

The early studies of Axelrod (Axelrod *et al.* 1959, Whitby *et al.*, 1961) and later of Iversen (1963, 1965) have established that uptake of NA by active membrane transport of the amine into the cytoplasm of the nerve is a major mechanism for removing the amine from the region of the synaptic cleft. Some features of this process relevant to the present study are -

1) that it is saturable with a K_m of 0.27×10^{-6} mol l^{-1} NA in rat heart.
2) That it is probably stereospecific in the rat although not in the guinea pig or rabbit.

The evidence in the rat is that the initial rate of uptake of lNA exceeded that of dNA in reserpinized rat heart (Iversen *et al.*, 1971). However no preference was shown for lNA in the guinea pig heart. The evidence in the rabbit is that Draskoczy & Trendelenburg (1968) found no difference in the initial rate of uptake of d and lNA in the rabbit heart. Moreover, although the rabbit aorta accumulates more lNA than dNA within nerves in enzyme inhibited preparations, this appears to be due to a preference for the l isomer displayed by vesicle binding, transport into the axoplasm showing no stereoselectivity (Eckert *et al.*, 1976).

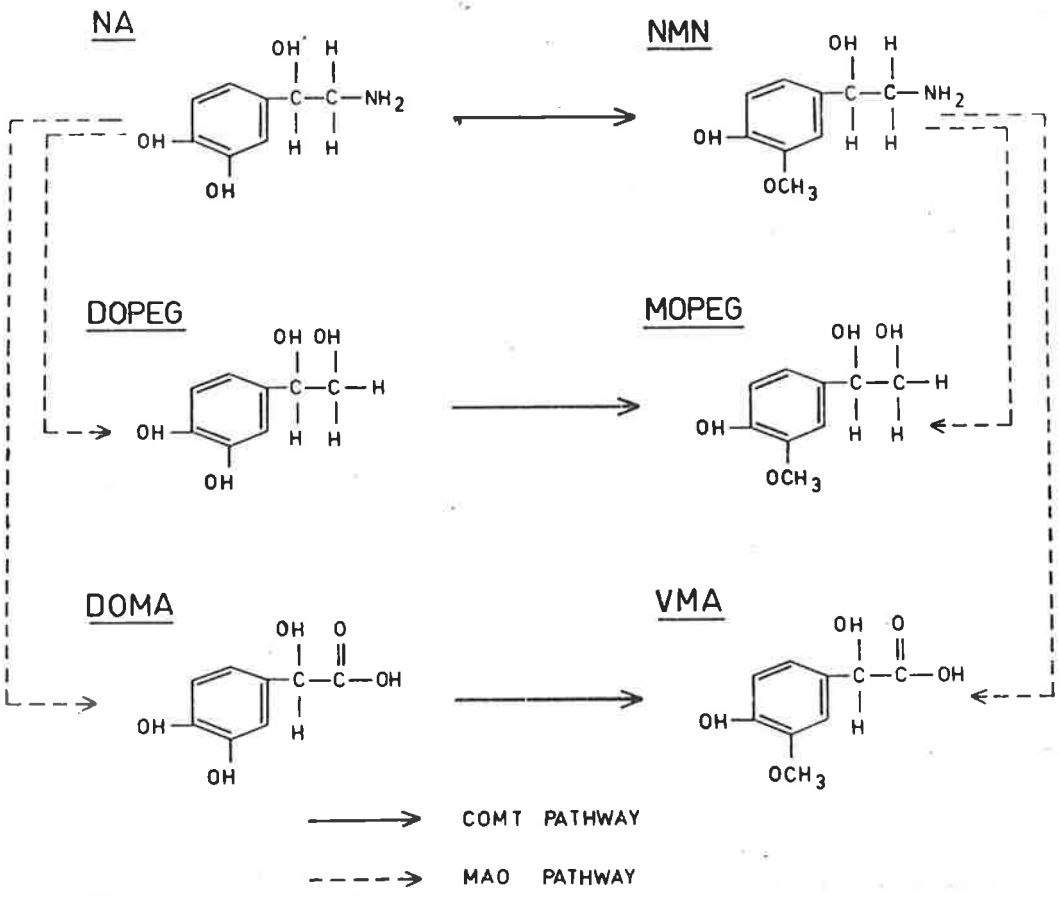

FIGURE 1.1. Major pathways for the metabolism of NA in tissues, ——→ COMT, - - - - → MAO.

3) that it is inhibited by cocaine. Evidence for a neuronal action of cocaine is both pharmacological and biochemical.

(i) Pharmacological evidence for a neuronal action of cocaine is constituted by the fact that potentiation of NA responses by cocaine is markedly reduced or abolished in tissues in which the adrenergic nerve terminals have degenerated after denervation (Langer *et al.*, 1967a, cat nictitating membrane; de la Lande *et al.*, 1967, rabbit ear arter; Green & Fleming, 1968, cat spleen). In the cat nictitating membrane cocaine causes a greater supersensitivity to NA than to adrenaline and has no effect on sensitivity to methoxamine (Trendelenburg *et al.*, 1970) an effect which correlates with the affinities of these amines for neuronal uptake (Draskoczy & Trendelenburg 1970). The degree of sensitization caused by cocaine is also inversely related to the neuromuscular interval (Verity 1971) which agrees with the influence of neuronal uptake decreasing as the distance between uptake and receptor sites increases. Thus de la Lande *et al.* (1967b) showed that cocaine potentiated extra luminally applied NA in the rabbit ear artery but had little effect on intra-luminally applied NA, which can be explained by the fact that the nerves are located on the extraluminal surface of the ear artery. However there is one example of post junctional potentiation of amines by cocaine. Cocaine has been shown to potentiate the response of rabbit aortic strips to methoxamine (Kalsner & Nickerson 1969) an amine which is not taken up by sympathetic nerves (Trendelenburg *et al.*, 1970). Cocaine also potentiated responses to NA in rabbit aortic strips from which the nerves had been removed (Maxwell & Eckhardt 1972). Nevertheless the post junctional effect of cocaine

in this tissue is small (approximately 2 fold potentiation) and a similar effect has not been demonstrated in other tissues.

(ii) Biochemical evidence: Early histochemical studies demonstrated that cocaine blocked the neuronal uptake of NA in the rat iris (Hillarp & Malmfors 1964). The selectivity of cocaine's effect on neuronal uptake of NA has been demonstrated in a number of studies of its effect on uptake and metabolism of exogenous NA. In such studies there is increasing evidence that the main metabolite produced by sympathetic nerves is 3,4 dihydroxyphenylglycol (DOPEG). In the rabbit aorta Levin (1974) demonstrated that the adventitia which contains the nerves forms mainly DOPEG while the media, devoid of nerves, formes mainly normetanephrine (NMN). In the rabbit ear artery, the major metabolite is DOPEG and this is the only metabolite significantly reduced in denervated arteries (Head *et al.*, 1975). In the rat vas deferens which is densely innervated Graefe *et al.* (1973) identified DOPEG as the major metabolite of NA. In the cat nictitating membrane, Langer & Enero (1974) demonstrated that the major metabolite spontaneously released was DOPEG and that this was the only metabolite to increase during nerve stimulation.

In the rabbit ear artery Head (1976) demonstrated that cocaine and denervation had similar effects on the accumulation and metabolism of ^{3}H NA and cocaine had no significant effect on metabolite formation in denervated preparations. Cocaine inhibited DOPEG formation by more than 90% in solutions bathing ear arteries but did not decrease the formation of other metabolites (de la Lande *et al.*, 1978). In the rabbit aorta, Henseling *et al.* (1976) identified neuronal and extraneuronal compartments for the uptake of ^{3}H NA.

Cocaine inhibited the uptake of ^{3}H NA only into the compartments identified as neuronal. Langer & Enero (1974) showed that only DOPEG was reduced by cocaine in the stimulated cat nictitating membrane and Head *et al.* (1975) demonstrated virtually no spontaneous release of ^{3}H from rabbit ear arteries when cocaine was present during preloading of the tissue with ^{3}H NA.

Following neuronal uptake, NA is bound in vesicles which limits its metabolism by neuronal MAO (Kopin *et al.*, 1962). The metabolism is considered subsequently.

NA may also be taken up by extraneuronal tissues. The main features of the extraneuronal uptake processes have been defined by Gillespie (1968, 1973) who studied the process at high substrate concentrations and by Trendelenburg *et al.* who studied the processes at low substrate concentrations (Bonisch *et al.*, 1974, Trendelenburg 1976). The main features of extraneuronal uptake relevant to this thesis are -

1) that it is saturable, having a high capacity for accumulating catecholamines but a low affinity (K_m for NA accumulation in rat heart = 2.52×10^{-4} mol l^{-1} Iversen 1965).

2) that it is not stereospecific (Iversen 1965).

3) that it shows species variation, being greatest in mouse and rabbit and least in guinea pig (Gillespie & Muir 1970).

4) that extraneuronally accumulated amines are rapidly removed from the tissue on washing (Gillespie 1968).

5) that it is not inhibited by cocaine but is inhibited by haloalkylamines such as phenoxybenzamine (Eisenfeld *et al.*, 1967), by

o-methylated metabolites of catecholamines such as NMN (Iversen 1965) and by various steroids including 17β oestradiol and progesterone (Iversen & Salt 1970).

6) there are a number of compartments involved in extraneuronal uptake of catecholamines. The rat heart has a high affinity, low capacity compartment for the o-methylation of isoprenaline (ISO) and a low affinity high capacity compartment for storing unchanged amine, both of which are steroid sensitive (Bonisch *et al.*, 1974). In the cat nictitating membrane there is a similar high affinity, low capacity, o-methylating compartment for ISO and NA which is steroid sensitive but there is also a non-steroid sensitive o-methylating compartment of different affinity from the steroid sensitive one.

Salt (1972) demonstrated that the IC 50 for inhibition of extraneuronal uptake in the perfused rat heart was 2.0×10^{-6} mol l^{-1} 17β oestradiol and 33.6×10^{-6} mol l^{-1} progesterone. 17β oestradiol was also a weak inhibitor of neuronal uptake but progesterone had little effect. Salt (1972) suggests that these steroids may influence vascular sensitivity to catecholamines by inhibition of extra-neuronal uptake in late pregnancy. Although the IC 50 values are approximately 10 times higher than the circulating levels of these steroids in pregnancy (Bedford *et al.*, 1962) it is possible that their combined action may influence extraneuronal uptake in the manner suggested by Salt (1972).

The uptake processes for NA have been shown under certain circumstances to determine the concentration of NA at adrenoceptors. In cat nictitating membrane, degeneration of sympathetic nerve

terminals (van Orden *et al.*, 1967) and a decrease in the ability of the degenerating nerves to retain exogenous NA (Smith *et al.*, 1966) develop at the same time as denervation supersensitivity to NA. There is also evidence that cocaine which inhibits neuronal uptake produced a prejunctional supersensitivity in densely innervated tissues such as the cat nictitating membrane (Trendelenburg *et al.*, 1972) which is unrelated to any post-junctional effect of cocaine. Under certain circumstances, extraneuronal uptake may also be important in determining sensitivity of a tissue to NA. Kauman (1972) showed that hydrocortisone caused supersensitivity of the cat papillary muscle to NA in the presence of cocaine and at low substrate concentrations, as did inhibition of COMT. The effects of hydrocortisone and COMT inhibition were not additive suggesting the compartment for NA uptake which was hydrocortisone sensitive was intimately associated with COMT, as demonstrated in the cat nictitating membrane by Graefe & Trendelenburg (1974).

(b) Monoamine Oxidase

MAO is a group of enzymes which oxidatively deaminate aliphatic and aromatic amines to their corresponding aldehydes. MAO was originally shown to deaminate A in the liver by Blaschko *et al.* (1937) who studied its substrate specificity. The enzyme has been shown to be located in tissues in the mitochondria (Hawkins 1952, De Lores Arnaiz & De Robertis 1962) in particular in the outer mitochondrial membrane (Greenwalt & Schnaitman 1970). In most species it shows no stereochemical specificity for d or l isomers of A or NA (Pratesi & Blaschko 1959) although the initial rate of oxidation of l-A in guinea pig liver exceeds that of d-A. Inhibitors of MAO have been reviewed by

Ho (1972) and Houslay & Tipton (1974) and include the irreversible inhibitors, nialamide and pargylene, which have been used extensively in biochemical and pharmacological studies of MAO.

There is considerable evidence that MAO exists in multiple forms exhibiting different physicochemical properties including different substrate specificities (Youdim *et al.*, 1969) different electrophoretic mobilities (Youdim & Sandler 1967) and different thermal stabilities (Youdim & Sourkes 1965). Differences in sensitivities to inhibitors have also been described. Johnston (1968) using rat brain MAO and tyramine as a substrate, described selective inhibition of type A MAO as opposed to type B by clorgylene. Type B has been shown to be selectively inhibited by deprenil (Knoll & Magyar 1972). Subsequently, these two types of MAO, A & B, have been associated with different substrate specificities A preferentially metabolizing NA and 5HT (Hall *et al.*, 1969) while B has a higher affinity for β phenylethylamine (Yang & Neff 1973) and benzylamine (Houslay & Tipton 1974). Tyramine, tryptamine, kynuramine and dopamine are metabolized by both forms of the enzymes (Squires, 1972, Houslay & Tipton 1974). Although type A and B MAO cannot be related to the electrophoretically separated isoenzymes described by Youdim & Sandler (1967), Dias Borges & D'Iorio (1973) have achieved electrophoretic separation of type A and B MAO from rat brain. More recently, the use of clorgylene and deprenil to distinguish type A & B MAO in the perfused lung (Bakhle & Youdim 1975) has eliminated the possibility that these isoenzymes of MAO are artefacts of the solubilization procedures used in the preparation of purified forms of MAO.

MAO has been shown histochemically to be associated with both sympathetic nerve cells (Dahlstrom *et al.*, 1969) and with extra-neuronal cells in the rat salivary gland (Almgren *et al.*, 1966) and rabbit ear artery (de la Lande *et al.*, 1970). Goridis & Neff (1971a & b, 1973) have demonstrated an association of type A MAO with nerve cells and type B with extraneuronal tissue in that rat superior cervical ganglia contain a high proportion of type A while the pineal gland contains a high proportion of type B. Denervation caused a selective loss of type A MAO in rat arteries (Coquil *et al.*, 1973) and in rabbit and rat vas deferens (Jarrott & Langer 1971). However, it is still not known to what extent both types of enzymes may be present within the same cells.

Although MAO catalyses the formation of aldehydes from monoamines, in the intact tissue these are intermediate products, being converted to the corresponding acid and alcohol products. The mitochondrial enzyme, aldehyde dehydrogenase catalyses the formation of the acid product (Erwin & Deitrich 1966) while the soluble enzyme, aldehyde reductase catalyses the formation of the alcohol product in bovine brain (Tabakoff & Erwin 1970).

There is some evidence that the aldehyde reductase displays a selectivity for the l isomer of NA (Levin 1974, Graefe *et al.*, 1973).

(c) COMT

COMT catalyses the 3-o-methylation of catecholamines using the methyl group donor, s-adenosyl methionine. It was discovered and described by Axelrod & Tomchick (1958). The properties of COMT have been reviewed by Guldberg & Marsden (1975). The liver enzyme is not stereochemically specific having similar affinities for d and l NA

(Axelrod & Tomchick 1958). There is evidence for multiple forms of the enzyme but their functional significance is uncertain (Guldberg & Marsden 1975). Soluble COMT from rat liver has been shown to consist of 2 or 3 fractions after electrophoresis (Axelrod & Vessell 1970). The enzyme is mainly cytoplasmic and soluble but in some cases particulate COMT has been described e.g. in the rat erythrocyte (Bohuon & Assicot 1973).

Inhibitors of COMT includepyrogallol and its derivatives, catechols and compounds isoteric with catechols. Inhibitors used in pharmacological studies include 3,4 dihydroxy 2-methyl propiophenone (UO521), pyrogallol and tropolone. Phenoxybenzamine and cocaine are not inhibitors of COMT (Eisenfeld *et al.*, 1967, Holtz *et al.*, 1966) but ascorbic acid is a weak inhibitor (Blaschke & Hertting 1971). The 2-hydroxylated oestrogens have been shown to be competitive inhibitors of rat liver COMT *in vivo* and *in vitro* although the parent oestrogens are without effect on COMT activity (Knuppen *et al.*, 1969). The parent oestrogens may however influence o-methylation of catecholamines in intact tissue by virtue of inhibiting extraneuronal uptake of catecholamines (Iversen & Salt 1970).

In most tissues COMT appears to be located mainly extraneuronally. The evidence for extraneuronal localization is indirect in that most tissues do not show a decrease in COMT activity after chronic denervation, exceptions being rabbit and rat vas deferens and cat nictitating membrane (Jarrott 1971b). In cat nictitating membrane

Jarrott & Langer (1971) demonstrated both neuronal and extraneuronal COMT in homogenates. There is also some evidence that ^{3}H NA is o-methylated at both neuronal and extraneuronal sites in the isolated cat nictitating membrane (Langer 1970) although NMN appears to be found mainly extraneuronally (Langer *et al.*, 1972).

In recent years chromatographic techniques have become available for separating the metabolites of NA from tissues. These include paper chromatography (Levin 1974) and ion exchange chromatography (Graefe *et al.*, 1973). Their use in conjunction with high specific activity radiolabelled catecholamines has enabled detailed studies of the metabolism of NA by isolated tissues. As discussed above, such studies have led to increasing evidence for a neuronal origin of DOPEG, whereas in both rabbit aorta (Levin 1974) and ear artery (Head *et al.*, 1975) o-methylated metabolites have been shown to be produced extraneuronally.

(d) Inactivation of NA by the Uterus

Relatively little is known of the mechanisms of inactivation of catecholamines in the uterus. In 1964, Wurtman, Axelrod & Potter demonstrated COMT and MAO activity in homogenates of rat uterus, MAO activity greatly exceeding that of COMT. COMT activity increased two fold during pregnancy while MAO activity was unaltered.

The activity of both enzymes has been shown to alter with the stage of the oestrous cycle in the rat. However the data for uterine COMT activity is conflicting in that Giles & Miller (1967) demonstrated greater COMT activity in uteri during oestrous than

dioestrous while Salseduc *et al.* (1966) demonstrated less COMT activity during oestrous than during dioestrous. However Green and Miller (1966a) reported an increased formation of o-methylated metabolites of A in uterine slices from oestrous rats compared with dioestrous rats.

Southgate *et al.* (1968) demonstrated histochemically and biochemically that human endometrial MAO activity was low during the proliferative stage of the menstrual cycle and high during the secretory stage. Southgate *et al.* (1969) subsequently showed that whole rat uterine MAO activity was increased after short-term progesterone treatment in ovariectomized rats while it was decreased after similar treatment with 17β oestradiol. Kynuramine was used as the substrate in this study but subsequently similar results were obtained using tyramine, tryptamine and benzylamine as substrates although progesterone produced a greater increase in MAO activity with dopamine as substrate while oestrogen was without effect (Southgate 1972). Collins and Southgate (1970) showed that rat uterine MAO was competitively inhibited by 17β oestradiol *in vitro* but it is difficult to assess the significance of this effect *in vivo*. These same authors (Collins & Southgate 1970) demonstrated that on separation of rat uterine MAO into three bands by electrophoresis, one of the bands, designated MAO III, showed changes in activity after oestrogen and progesterone treatment similar to that observed in the straight homogenates. In view of the fact that similar results were obtained using substrates for both A & B types MAO (Southgate 1972) it seems unlikely that MAO III can be associated with either of the A or B isoenzymes described by Johnston (1968) although this has not been

tested. Subsequently the effects of these two steroids on rat uterine MAO activity has been confirmed by Holzbauer & Youdim (1973) using kynuramine as a substrate. Moreover, these authors demonstrated a variation in MAO activity with the stages of the oestrous cycle, uterine MAO being lowest during the night between oestrous and metoestrous and highest during metoestrous. The effect was not specific for the uterus since cyclical variation in ovarian, adrenal and hypothalamic MAO was also observed, peak MAO activity in the adrenal gland corresponding with its peak secretion of progesterone. These authors have demonstrated the need to sample rats during the dark stage of their light-dark cycle since lowest MAO activities were detected at this time.

In summary, rat and human (endometrial) uterine MAO activity increases with progesterone treatment or progesterone dominance and decreases with oestrogen treatment or oestrogen dominance. The mechanism of these effects has not been established.

Little information is available on the neuronal and extraneuronal location of MAO or COMT in uterine tissue. In 1966, Green & Miller investigated the accumulation of ^{3}H NA and ^{3}H A by slices of rat uterus *in vitro*. Green and Miller (1966a) demonstrated that the accumulation of ^{3}H A and ^{3}H NA was inversely related to the size of the rat uterus but relatively constant when expressed per uterus. The uptake was saturable at 2×10^{-5} mol l^{-1} NA and the uptake of A (2×10^{-9} mol l^{-1}) was inhibited by 90% by 4×10^{-5} mol l^{-1} cocaine. Presumably at this substrate concentration, most of the accumulated A and NA were intraneuronal which is also suggested by their later finding

(Green & Miller 1966b) that 80% of the radioactivity was retained in the tissue 60 minutes after washing with amine-free solution. This contrasts with the data of Wurtman *et al.* (1964) which indicated that A was bound at a different site from NA in the rat uterus. The discrepancy may be due to differences in substrate concentration since Wurtman *et al.* (1964) infused A into the rat *in vivo*.

Green & Miller (1966a) were unable to distinguish individual metabolites of NA although o-methylated metabolite fractions of A were isolated from mature rat uteri after incubation with ^{3}H A using alumina chromatography. No metabolites of A were detected from immature uteri. Uterine slices from oestrous animals formed significantly more o-methylated metabolites than those from dioestrous animals.

With the refinement of chromatographic techniques for separating metabolites of NA, it is now possible to investigate more fully the metabolism of ^{3}H NA in isolated uteri. Although the rat has been the most extensively studied animal in this respect, it has a fairly unique innervation in that it is mainly confined to the vasculature (Falck *et al.*, 1974). Moreover it is the only animal studied which has relatively high levels of endogenous A. For this reason, it was decided to study the uptake and metabolism of NA in the rabbit uterus, in which the innervation has been extensively described by Owman & Sjöberg (1966). No information has been published on uterine MAO or COMT activity in the rabbit nor on the metabolites formed by these two enzymes in the intact rabbit uterus. The aims of this thesis were -

1) to quantitate the metabolites of NA formed by the rabbit uterus *in vitro* and as far as possible to establish their neuronal or extraneuronal sites of formation.

2) to investigate the effects of pregnancy and the two ovarian steroids, oestrogen and progesterone, on the metabolism of NA by the rabbit uterus.

3) it was hoped that defining any such changes in this way might help to provide an insight into the function of the adrenergic innervation of the uterus.

CHAPTER 2.

GENERAL METHODS

1. EXPERIMENTAL ANIMALS

Sexually mature, nulliparous, semi-lop-eared rabbits, between 1.8 and 3 Kg. body weight were used in this study. All animals were bred at the central animal house, University of Adelaide. They were maintained on a positive cycle of 12 hours light, 12 hours dark.

For the studies on pregnancy, the rabbits were mated in the departmental animal house. Female rabbits were housed in individual cages and their mating with a buck observed and recorded as day 1 of pregnancy.

2. OVARIECTOMY

The rabbits were anaesthetized with sodium pentabarbitone (60.5 mmol. 1^{-1}) in sterile saline, by slow injection into a marginal ear vein until surgical anaesthesia was obtained. Surgical procedures were performed under sterile conditions. The ovaries were approached through two flank incisions. Having located the ovaries they were isolated by clamping a pair of curved forceps around them. The ovarian artery and vein and the oviduct were ligatured as close to the ovary as possible with surgical cat gut and the ovaries were removed. The peritoneum and body wall were then sutured. The stitches were removed after 6 days. One month was allowed for recovery at which time no inflammation or swelling was evident.

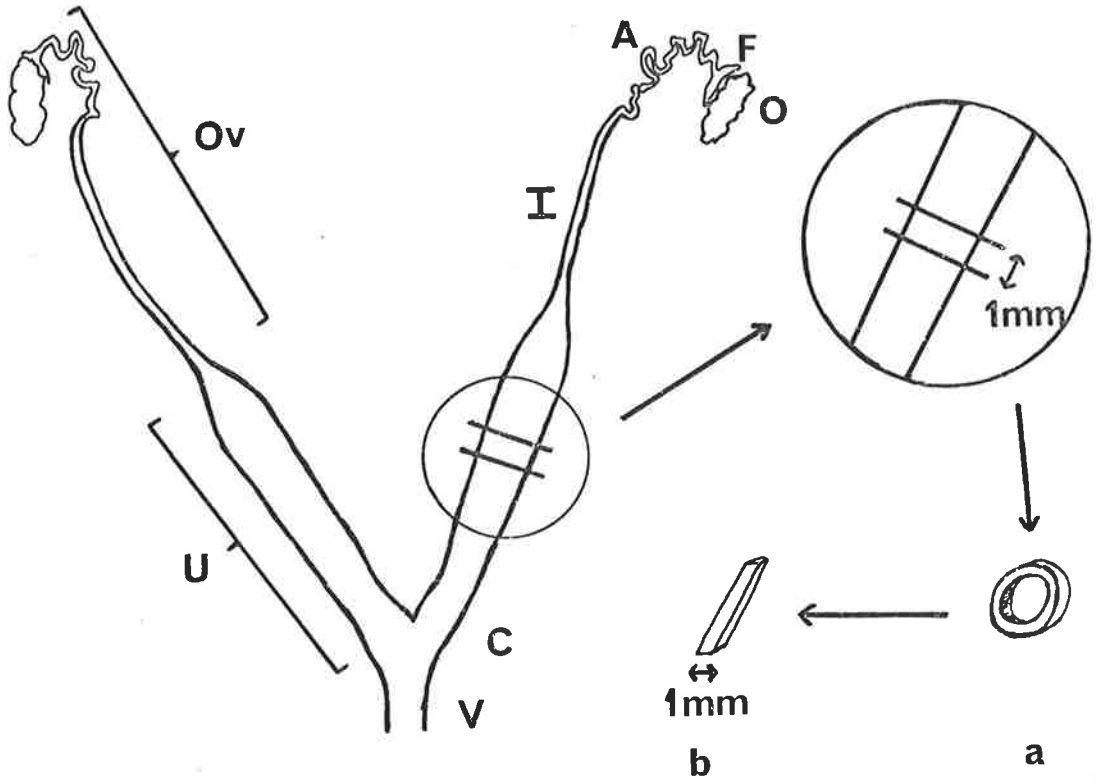

Fig. 2.1 Diagrammatic representation of reproductive tissues of the rabbit used for incubation studies. U = uterus, C = cervix, V = vagina, OV = oviduct, I = isthmus, A = ampulla, F = fimbrae, O = ovary. Uterine segments were prepared from the mid section of each uterine horn, a length of 1 mm being removed as shown.

3. ISOLATION OF TISSUES

The rabbits were killed by cervical dislocation and bled from the carotid arteries. The abdomen was opened and the reproductive tract located and quickly dissected free of fat and ligaments. For further dissection, the tissues were removed to beakers of warm Krebs bicarbonate solution gassed with 95% O_2/5% CO_2.

In experiments in which uteri were incubated with tritiated noradrenaline, segments of uterine tissue were prepared by transversely sectioning the uterine horn into rings approximately 1.0 mm thick. These sections were taken from the uterus approximately half way between the cervix and the utero-tubal junction. Each ring of uterine tissue was then cut open to produce a segment of approximately 30-40 mg wet weight (fig. 2.1). The oviducts were removed intact and included the utero-tubal junction. Ovaries were sectioned longitudinally before incubation.

In some experiments the central ear artery of the rabbit was also used for incubation studies. The artery was dissected free of fat and connective tissue and a length of approximately 3 cm proximal to the bifurcation was removed (fig. 2.2). The lumen was flushed with Krebs solution to remove any blood and the artery was transferred to warm gassed Krebs solution.

Prior to weighing, the tissues were blotted lightly on filter paper moistened with Krebs solution. They were weighed on a piece of parafilm on the weighing pan of a balance and then placed in Krebs solution at 36^{o}C and gassed with 95% O_2/5% CO_2.

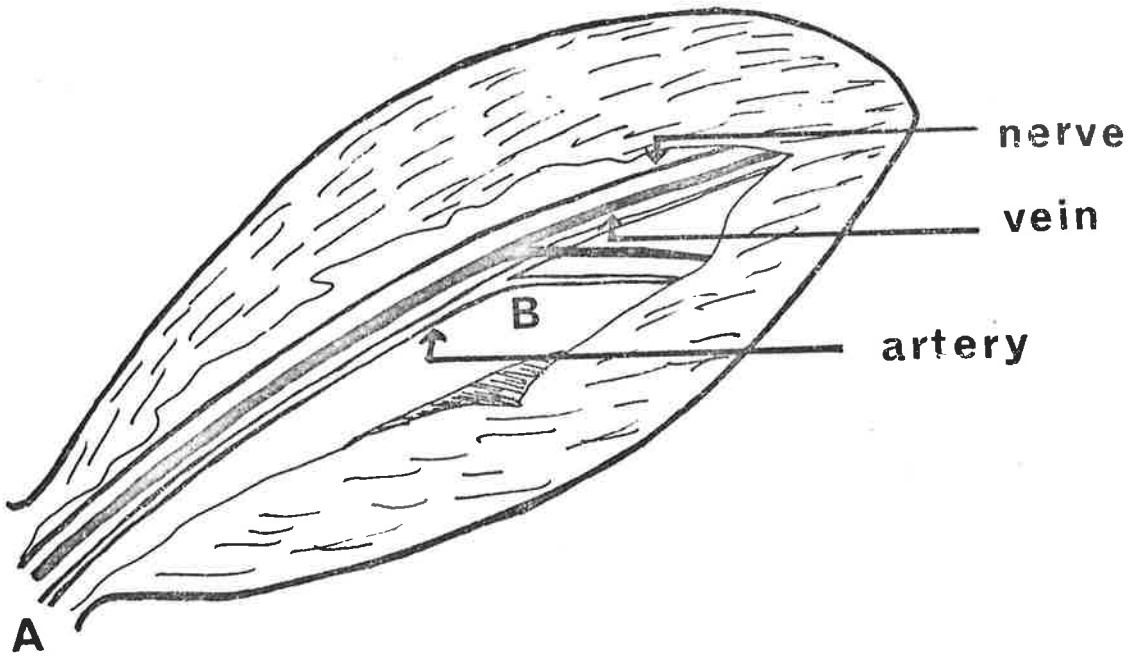

Fig. 2.2 Diagrammatic representation of the central artery of the rabbit ear. A length (AB) proximal to the bifurcation was used for incubation studies.

4. INCUBATION OF ISOLATED TISSUES

The same procedure was used for uteri and ear arteries. The incubation medium in all experiments with isolated tissues was Krebs bicarbonate solution, the composition of which is given in appendix 4. The solution was gassed with 95% O_2/5% CO_2. Ascorbic acid (290 μmol l^{-1}) was added to the incubation medium as an anti-oxidant, after the Krebs solution had equilibrated with the 95% O_2/5% CO_2 and reached a stable pH of 7.4. The tissues were incubated in glass vials (40 mm x 10 mm, internal diameter 8 mm) which were fitted individually with polyethylene tubing of small internal diameter to provide a continuous supply of gas (fig. 2.3). The vials were held in parallel rows in a rack fitted into a water bath which maintained the temperature thermostatically at 36°C. Pre-incubation media, incubation media and wash solutions were held in different vials and the tissues were transferred from one solution to the next using fine-tipped forceps. Drugs which were added to the incubation media were added in a volume not exceeding 0.02 ml/1 ml of incubation medium. In order to remove any tritiated material adhering to the outside of the tissues at the end of the incubation procedure, they were washed briefly in Krebs solution. After washing, they were blotted lightly on filter paper moistened with Krebs solution prior to extraction of tritiated compounds. The incubation procedure was usually complete within one and a half hours after removal of the tissues from the animals.

Uterine segments were incubated in 1.0 ml of Krebs bicarbonate solution.

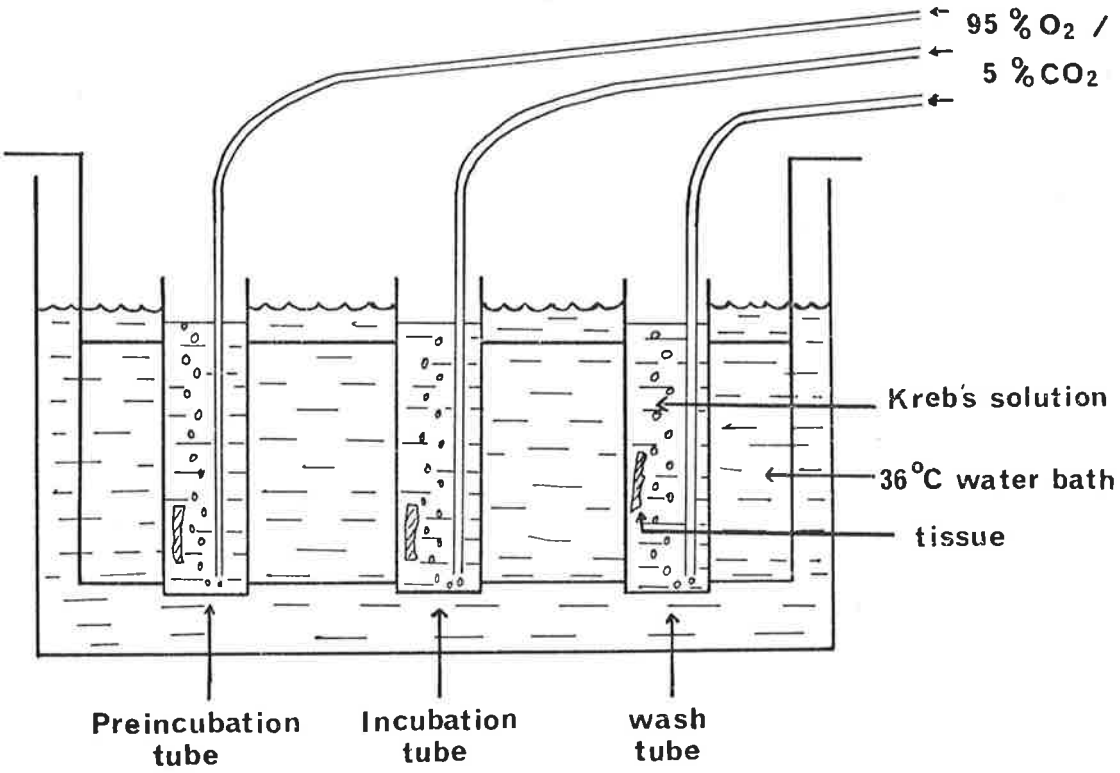

Fig. 2.3 The apparatus used for incubation of tissues with ^{3}H NA. Preincubation, incubation and wash solutions were contained in separate tubes as shown.

5. ENZYME ASSAYS OF TISSUE HOMOGENATES

One uterine horn (excluding the utero-tubal junction) was removed as described above and immediately placed into a tube of iced sodium phosphate buffer. After weighing on parafilm, the uteri were homogenized in phosphate buffer (5 mmol. l^{-1}, pH 7.0) using a 1/10 weight to volume dilution, with an Ultra Turrax homogenizer (Janke & Kunkel). The samples were kept on ice during homogenization.

Assays for COMT were performed on the crude homogenate. Assays for MAO were performed on partially purified homogenates. The crude homogenate was centrifuged at 900 g for 30 mins at $4^{o}C$ to remove cell debris. The resulting supernatant was removed and centrifuged at 10,000 g for 30 mins at $4^{o}C$, after which the pellet was washed by resuspending in phosphate buffer and centrifuged again at 10,000 g for 30 mins. The washed pellet was resuspended in sodium phosphate buffer to a volume equal to the initial volume centrifuged and used for assay. Fig. 2.4 summarizes the treatment of tissue homogenates.

(a) Monoamine Oxidase Assay

The method of Jarrott (1971a) which utilizes ^{3}H tyramine as a substrate was used for this assay. The reaction mixture consisted of 0.025 ml of tissue homogenate (re-suspended 10,000 g pellet), 0.025 ml of water and 0.05 ml of sodium phosphate buffer (200 mmol

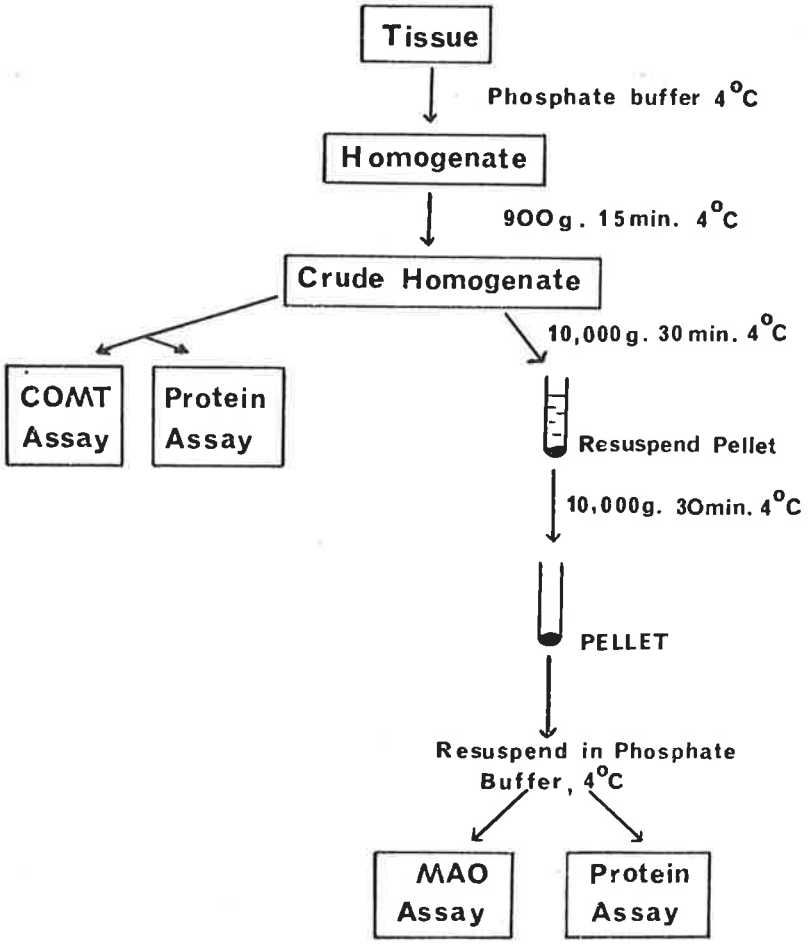

Fig. 2.4 Preparation of tissue homogenates for assays of MAO and COMT activity.

1^{-1}, pH 7.8) containing ^{3}H tyramine (2 mmol 1^{-1}, 20 μCi/μmol). Blanks were prepared by replacing the tissue homogenates with 0.025 ml of sodium phosphate buffer (5 mmol 1^{-1}, pH 7.0). The reaction was carried out at 36°C in oxygen-filled glass centrifuge tubes (internal diameter 10 mm tapering to 4 mm at the bottom) with continuous shaking at 100 oscillation per min for 30 min. The reaction was stopped by the addition of 0.01 ml of HCl (3 mol 1^{-1}) to the tubes which were then placed on ice. The product was extracted into ethyl acetate as follows: 0.5 ml of ethyl acetate was added to the tubes which were shaken for 5 min. They were then centrifuged for 5 min to separate the two phases and 0.4 ml of the organic phase was transferred to centrifuge tubes containing 0.1 ml of HCl (3 mmol 1^{-1}) and shaken for a further 5 min. After centrifugation at 2,000 g for 5 min, 0.3 ml of the organic layer was removed and added to scintillation vials containing 15 ml of Toluene-based scintillant. The ^{3}H contents were measured by liquid scintillation spectrometry (see section 7). The enzyme activity was expressed as nmol of product formed per mg protein per hour. All assays were performed in duplicate and the enzyme activity expressed as the mean of two determinations. The reaction proceeded linearly with time of incubation up to 30 mins and the reaction velocity was maximal at the concentration of tyramine used.

(b) Catechol-O-Methyl Transferase Assay

COMT was assayed by the method of Jarrott (1971b). This is essentially a modification of the method reported by Mc Caman (1965).

The reaction mixture consisted of 0.05 ml of sodium phosphate buffer (0.16 mol l^{-1}, pH 7.8), 0.025 ml of tissue homogenate and 0.025 ml of water containing 3, 4, dihydroxy benzoic acid (4 mmol l^{-1}), ^{14}C S-adenosyl methionine (200 µmol l^{-1}, 30 µCi/µmol) and $MgCl_2$ (100 mmol l^{-1}). Blanks were prepared in a similar manner to those for the assay of MAO activity. The reaction was carried out in glass centrifuge tubes for 30 mins at 36°C with continuous shaking at 100 oscillations/min. It was stopped by the addition of 0.01 ml of HCl (3 mol l^{-1}) and the tubes were placed on ice. The product was extracted into ethyl acetate and the ^{14}C content of the ethyl acetate determined in a similar manner to that described for MAO assays.

The COMT activity was expressed as nmol of product formed per mg protein per hour and the activity of a given sample was the mean of two determinations. As in the case of the MAO assay, it was established that the reaction was linear with time of incubation and with enzyme concentration.

(c) Protein Estimation

The protein content of homogenates was determined by the method of Lowry *et al.*(1951). Fig. 2.5 summarizes the procedure used. Tissue homogenates were diluted 1/50 and 1/100 in sodium phosphate buffer (5 mmol l^{-1}, pH 7) and 1.0 ml added to glass test tubes. Standard solutions of bovine serum albumin in phosphate buffer in a concentration range of 10 to 100 µg ml^{-1} were also prepared and

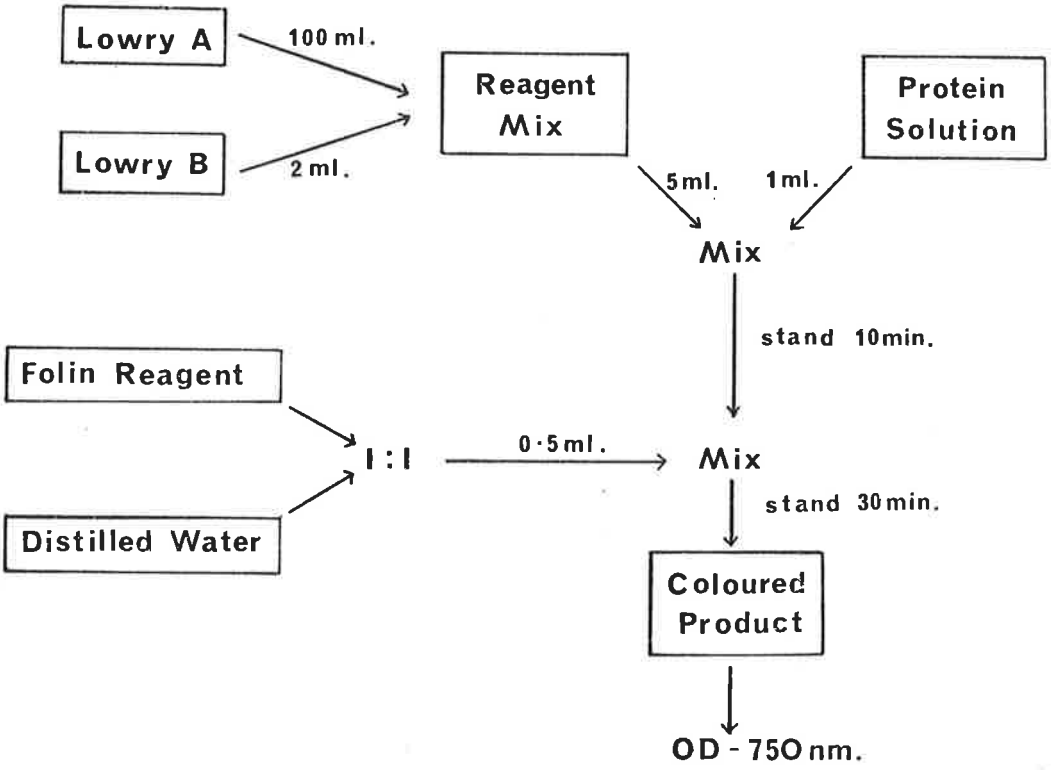

Fig. 2.5 Diagrammatic representation of the Lowry-Folin procedure for protein estimations.

added to test tubes in a volume of 1 ml. The reagent mixture consisted of 100 ml of Lowry A solution freshly mixed with 2 ml of Lowry B solution. Lowry A consisted of 217 mmol l^{-1} Na_2CO_3 in NaOH (100 mmol l^{-1}). Lowry B consisted of $CuSO_4$ (32 mmol l^{-1}) containing sodium potassium tartrate (41 mmol l^{-1}).

5.0 ml of the reagent mixture was added with rapid mixing to the protein solutions and left to stand for 10 mins. 0.5 ml of Folin reagent (commercial solution diluted 1:1 with distilled water) was then added with rapid mixing to all tubes. After 30 mins the OD at 750 n metres was measured using a Bausch and Lomb Spectronic 20 photometer. Determinations were made in duplicate and each sample was expressed as the mean of two determinations. A standard curve of OD versus protein concentration was constructed for each series of assays and the protein contents of the diluted homogenates were determined from this curve.

6. ASSAY OF ENDOGENOUS CATECHOLAMINE CONTENTS OF TISSUES

The noradrenaline and adrenaline contents of tissues were determined using the semi-automated trihydroxy indole assay described by Head *et al.*(1977). Tissues were weighed and placed into tubes containing HCl (100 mmol l^{-1}) and EDTA (30 µmol l^{-1}). The volume of HCl used was 10 ml/g of tissue. Uteri, ovaries and oviducts were sliced before placing in HCl to facilitate extraction. Ear arteries did not require slicing prior to extraction. The tissues were extracted for 20 hours at $4^{o}C$. Under these conditions

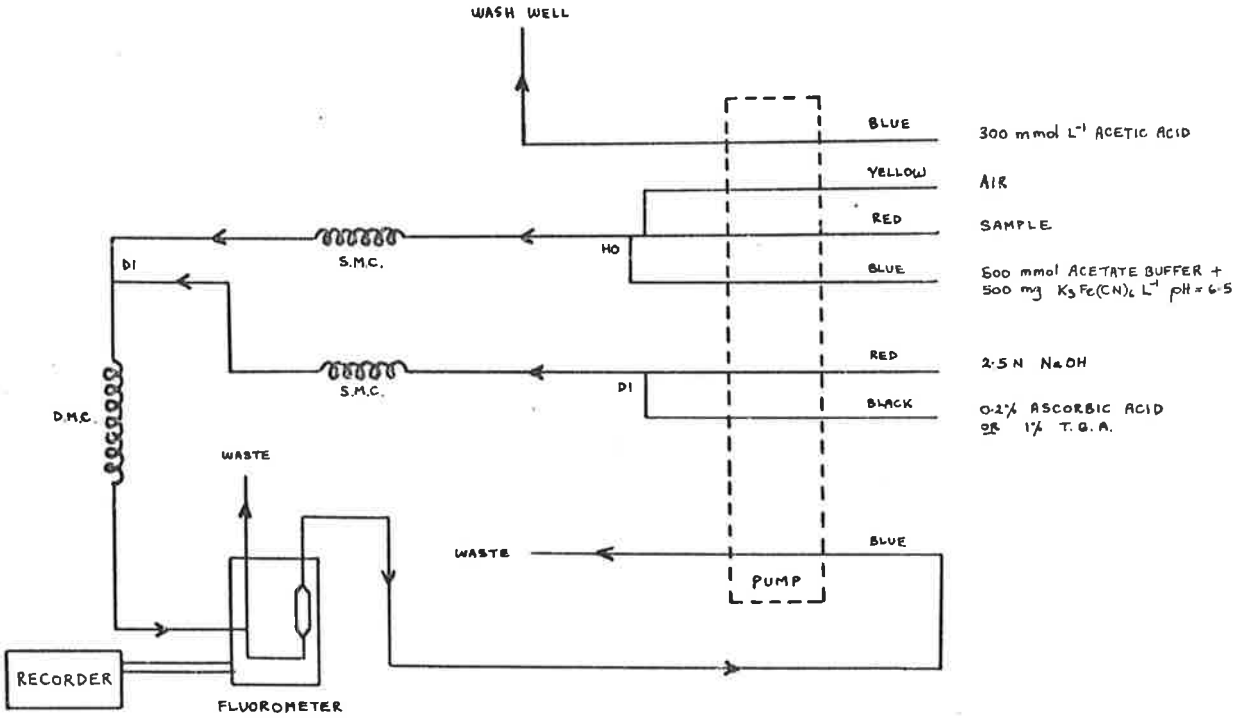

Fig. 2.6 A schematic representation of the automated fluorimetric assay for catecholamines as applied to samples eluted from alumina in 0.3 mol l^{-1} acetic acid.

96% of NA present in the tissue is extracted as estimated by extraction of ^{3}H NA. The extracts were purified immediately on alumina (de la Lande *et al.*, 1967) since storage at 4°C in 100 mmol l^{-1} HCl for 14 days resulted in a 5-10% loss of noradrenaline. The catecholamines were eluted from the alumina in 5 ml of 0.5 mol l^{-1} HCl and assayed fluorometrically employing the technicon autoanalyser manifold shown in fig. 2.6. ^{3}H l NA added to the samples prior to alumina purification provided an estimate of the recovery of NA. The mean recovery of NA after alumina chromatography was 74.1% ± 1.8 (n=33). When 0.2% ascorbic acid was used in the assay, total catecholamines were measured. When the ascorbic acid was replaced by 1% thioglycolic acid (TGA) only NA contributed to the development of a fluorescent product. Adrenaline concentration was obtained by the difference between the two estimates. All samples were corrected for non-oxidized blanks prepared by the method of Merrills (1963).

7. LIQUID SCINTILLATION SPECTROMETRY

A Packard Model 3310 Liquid Scintillation Spectrometer was used to measure the radioactivity present in solutions containing carbon-14 labelled (^{14}C) and tritium labelled (^{3}H) compounds. Aqueous solutions (0.1 to 1.0 ml) or ethyl acetate solutions (0.3 ml) were added to glass scintillation vials containing 15 ml of a toluene-based phosphor of the following composition: 8.25 g of 2,5-diphenyloxazole (PPO), 0.25 g of 1,4-bis-2-(5-phenyloxazolyl)-benzene (POPOP), and 500 ml of Triton X100 added to 1 litre of toluene. Correction for quenching and efficiency of counting was made by using internal

standardization. The internal standards were ^{3}H toluene or ^{3}H water (Packard) for samples containing ^{3}H, or ^{14}C toluene (Packard) for samples containing ^{14}C. A small volume of standard of known activity (A) was added to test samples after an initial rate count (r_1) had been made for these samples. The samples were then recounted and the second rate count recorded (r_2). The efficiency of counting (E) was then estimated from

$$E = \frac{r_2 - r_1}{A}$$

The activity of the test sample was then estimated from $\frac{r_1}{E}$.

The percentage efficiency of counting was 18-25% for ^{3}H and 60-70% for ^{14}C. The limit of detectability of ^{3}H in solutions was derived from

$$\frac{2}{\frac{E^2}{r^b}}$$ where r_b = background rate count.

For a count period of 1 min and a 95% probability estimation this was approximately 100 DPM for most solutions.

CHAPTER 3.

SPECIFIC METHODS

This chapter describes the techniques used in the purification of tritiated noradrenaline for experiments on its accumulation and metabolism by tissues and the techniques for its subsequent separation from its metabolites after incubation with those tissues.

1. PURIFICATION OF ^{3}H 1-NORADRENALINE

In view of the problem of impurities in manufacturer's solutions of ^{3}H 1-NA, all stocks of this compound were purified before use using a batch alumina process which has been reported previously by de la Lande *et al.* (1967). An aliquot of the manufacturer's stock solution was added to 30 ml polypropylene tubes containing approximately 10 ml of HCl (1 mmol l^{-1}), 4.9 mmol of activated alumina from which the fines had been removed, 570 µmol of ascorbic acid and 270 µmol of ethylene diamine tetra acetic acid (EDTA). The solution in the tube was bubbled continuously with N_2 and adjusted to pH 8.4 using sodium carbonate solution (1 mol l^{-1} and 0.1 mol l^{-1}). The pH was maintained at 8.4 for 4 minutes after which the alumina was allowed to settle and the effluent decanted. The alumina was then washed twice with 10 ml of distilled water before eluting the catecholamine with acetic acid. The alumina was mixed with 5 ml of acetic acid (300 mmol l^{-1}) for 5 minutes after which 4.5 mls was decanted. A further 2 mls of acetic acid was used to wash the alumina for 5 minutes and the two acetic acid fractions were then pooled. After centrifugation to remove any fines which may

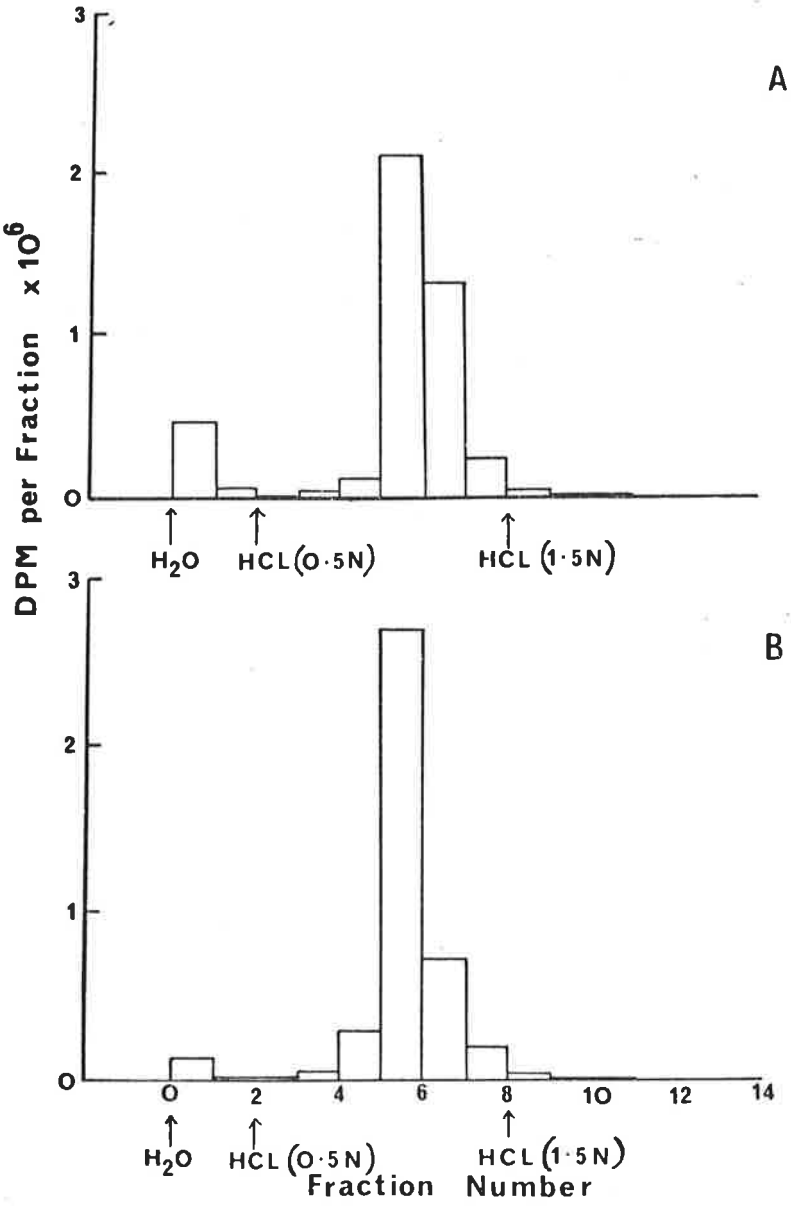

Fig. 3.1 Profile of radioactivity obtained by chromatography of ^{3}H 1-NA on Dowex 50 ion exchange columns. A: sample of ^{3}H 1 NA before and B: after purification on alumina.

still have been present the ^{3}H content of the acetic eluate was estimated by liquid scintillation spectrometry and the NA content was assayed by the fluorescent trihydroxy indole assay described in chapter 2. The purified stock was stored in polypropylene tubes at $4^{o}C$ until further use.

A typical comparison of the profiles of solutions of ^{3}H l-NA on Dowex 50 ion exchange columns before and after alumina purification is shown in fig. 3.1. The amount of tritium recovered from the effluent fraction was greatly reduced after alumina chromatography.

2. PREPARATION OF ^{3}H l-NORADRENALINE BEFORE INCUBATION

Since ^{3}H l-NA was present in acetic acid (300 mmol l^{-1}) after purification, the amount required for each incubation study was lypholized immediately prior to use in order to remove the acetic acid. The stock solution was snap-frozen in lyphilization vials using acetone-dry ice prior to freeze drying. After lypholization the NA was immediately reconstituted at the desired concentration in Krebs bicarbonate ringer which was pre-gassed with 95% O_2/5% CO_2 and contained ascorbic acid (290 µmol l^{-1}) and EDTA (11 µmol l^{-1}). Provided lypholization was complete within 1 hour, recovery of NA was greater than 95% as estimated by liquid scintillation spectrometry and the automated trihydroxy indole assay. However longer periods of lypholization did lead to further losses of catecholamines. The 2.5 hours required to lypholize tissue extracts in the experiments described in section 4.b

resulted in a further 10% loss of NA, when compared to recoveries from non-lypholized solutions.

3. EXTRACTION OF ^{3}H l-NORADRENALINE AND METABOLITES FROM TISSUES

The tritium was extracted from tissues after incubation by one of two methods depending on the subsequent procedure which was to be used to separate NA and its metabolites.

Method A: For extracts which were to be analysed by ion exchange chromatography (see section 4.a) the tissues were placed into 1 ml of HCl (100 mmol l^{-1}) containing EDTA (30 μmol l^{-1}) and extracted for 20 hours at $4^{o}C$.

Method B: Extracts which were to be analysed by thin layer chromatography were prepared by extracting tissues in 2.5 ml of acetic acid (1 mol l^{-1}) containing EDTA (30 μmol l^{-1}) for 20 hours at $4^{o}C$. Acetic acid was used instead of HCl for extraction since the latter caused artefacts on the chromatograms (see section 4.b).

In both methods, the extracts were removed and the tissues washed with a further 1 ml of acid. The two acid samples were then pooled for each tissue. Table 3.1 shows the efficiency of extraction of tritium from tissues using these two methods. The efficiency of extraction was calculated by solubilizing the tissues after extraction with New England Nuclear Cell Solubilizer (Levin, 1973) and determining the amount of tritium left in the tissues

TABLE 3.1 Efficiency of Extraction of ^{3}H from tissues incubated with ^{3}H INA.

EXTRACTION MEDIUM	% EFFICIENCY
HCl (100 mmol l^{-1})	96.4 ± 0.2 (n = 8)
Acetic Acid (1 mol l^{-1})	95.1 ± 0.2 (n = 8)

after extraction. The efficiency was then calculated from

$$\frac{T}{T+R} \times 100\%$$

where T = the tritium extracted from the tissue and R = the residual tritium in the tissue after extraction. It will be seen that acetic acid extraction was only slightly less efficient than HCl extraction.

4. SEPARATIVE TECHNIQUES FOR NORADRENALINE AND ITS METABOLITES

(a) Combined ion-exchange and Alumina Chromatography

In earlier experiments described in chapter 5, NA and its metabolites were separated using chromatography on Dowex 50 (Na^+) * ion exchange columns followed by fractionation of the effluent on batch alumina (fig. 3.2). The method is the same in principle as those reported by Taylor and Laverty (1969) and Graefe *et al.* (1973).

At pH 6.4, the bases, NA and NMN, bind to Dowex 50 and can be eluted separately using different strength acids. The neutral and acid metabolites of NA do not bind to the column and appear in the effluent. Of these metabolites, the two catechols, DOPEG and DOMA will bind to alumina at pH 8.4, while the non-catechols, VMA and MOPEG do not bind. DOPEG and DOMA may be further separated from each other since DOPEG can be eluted from alumina using acetic acid (300 mmol l^{-1}) while DOMA, which is more strongly bound, may be eluted using HCl (500 mmol l^{-1}).

* Dowex 50 (x8), 200-400 mesh.

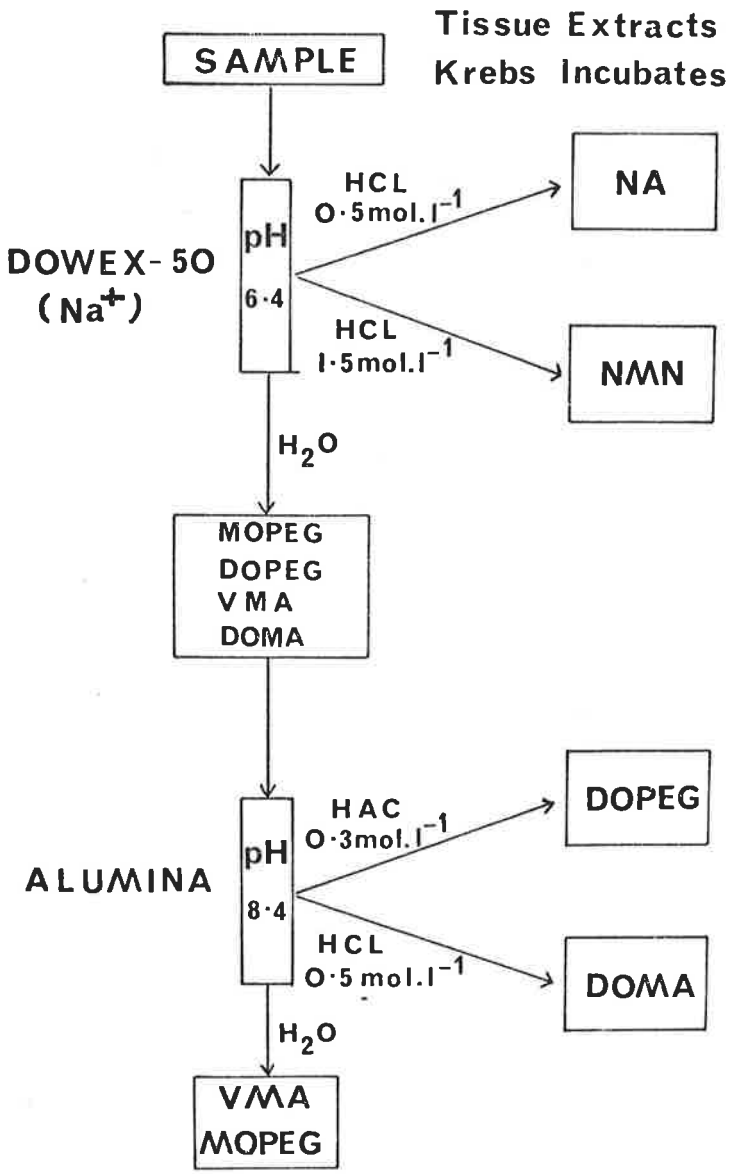

Fig. 3.2 Diagrammatic representation of column chromatography technique for separation of NA and its metabolites using Dowex 50 ion exchange columns and batch alumina.

(i) Ion Exchange Chromatography

Dowex 50 columns (Na^+, 200 - 400 mesh) were 4 mm in diameter and 60 mm in length. All test samples and solutions were pumped through the columns at a constant flow rate of 0.5 ml min^{-1} using a Technicon peristaltic pump. The test samples applied to the columns consisted of either 1.0 ml of incubation medium acidified with HCl (100 mmol l^{-1}) or 1.5 ml of tissue extract (100 mmol l^{-1} HCl). An anti-oxidant, sodium metabisulphite (53 µmol) was added to each sample followed by known quantities (1 µmol) of NA and each metabolite. The amount of unlabelled NA and metabolites present after chromatography provided an estimate of the recovery of tritiated compounds. The samples were adjusted to pH 6.4 with sodium phosphate buffer (100 mmol l^{-1}, pH 7.0) and loaded onto the columns. The effluent fractions, obtained by washing the columns with 15 ml of distilled water, were acidified immediately to pH 4.0 with HCl (5 mol l^{-1}) and stored at 4^oC until further separated on alumina. NA was eluted from the Dowex 50 columns in six 5 ml fractions with HCl (0.5 mol l^{-1}). NMN was subsequently eluted in three 5 ml fractions with HCl (1.5 mol l^{-1}). The ^3H content of 1.0 ml of each fraction was measured by liquid scintillation spectrometry and the recovery of NA or metabolites in each fraction was estimated using the colorimetric assay described in section 3.5. Co-chromatography of a peak of tritium with an unlabelled compound was taken as evidence for the existance of that compound in the tritiated test solution. Chromatography of Krebs incubating media containing ^3H l-NA but no tissue (incubate blanks) was used

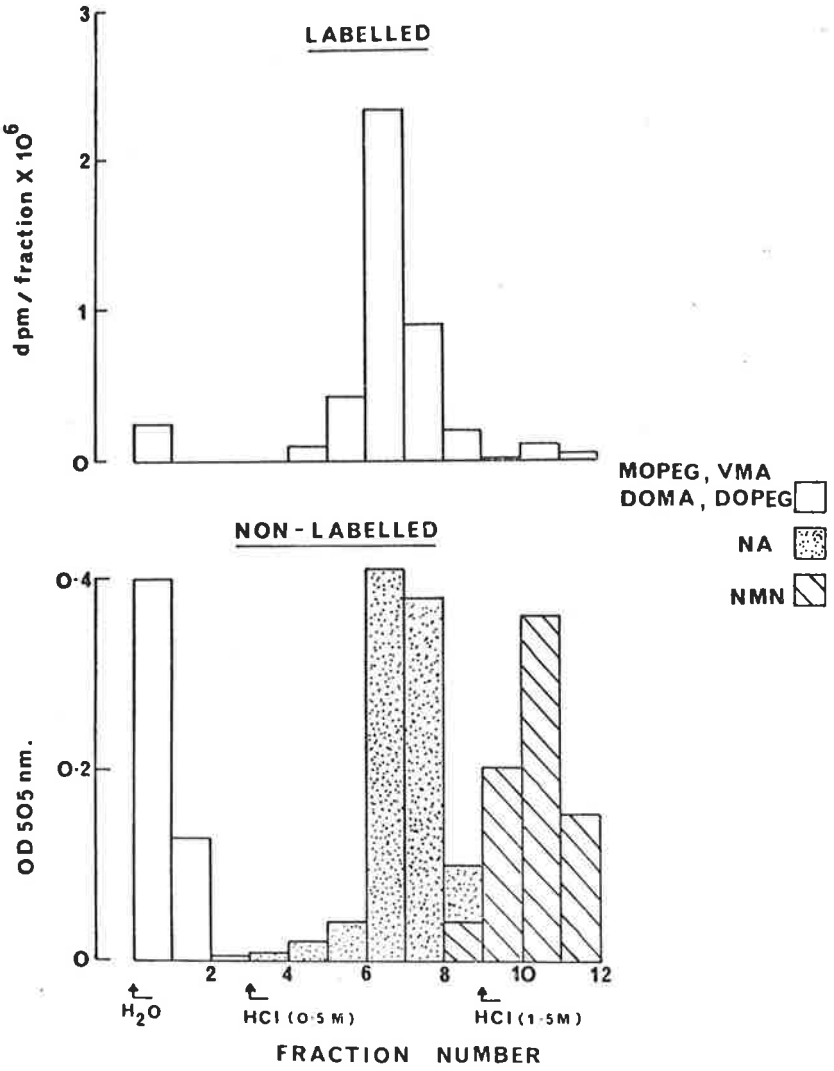

Fig. 3.3 Fractions obtained from Dowex 50 ion exchange columns. Upper panel: Profile of radioactivity after chromatography of incubating medium from tissue incubated with ^{3}H l NA. Lower panel: Profile of unlabelled metabolites of NA recovered from Dowex 50 columns. Unlabelled metabolites were applied separately to Dowex columns. Fractions were assayed by colorimetric method described in 3.5. VMA, MOPEG, DOMA and DOPEG were all found predominantly in fraction 1.

to indicate the degree of cross contamination of ^{3}H l-NA into other fractions. The separation of unlabelled NA and its metabolites on Dowex 50 and a profile of radio-activity found in fractions from Dowex 50 are shown in fig. 3.3.

(ii) Alumina Chromatography

The method used was similar to that described for the purification of ^{3}H l-NA except that the anti-oxidant used was sodium metabisulphite (53 μmol) instead of ascorbic acid. After bubbling with N_2 at pH 8.4 for 5 min, the effluent fraction was collected. This contained VMA and MOPEG and was not separated further. After two 10 ml washes with distilled water, DOPEG was eluted from the alumina by mixing it with 5 ml of acetic acid (300 mmol l^{-1}) for 5 mins. The alumina was then washed twice with 10 ml of acetic acid. DOMA was eluted subsequently by mixing the alumina with 5 ml of HCl (500 mmol l^{-1}) for 5 mins. In one series of experiments, DOPEG and DOMA were eluted together using 6 ml of HCl (500 mmol l^{-1}), omitting the prior acetic acid step. One ml of each fraction was used to estimate the ^{3}H content by liquid scintillation spectrometry while the recovery of unlabelled metabolites was estimated colorimetrically (see section 3.5). Fig. 3.4 shows the separation of unlabelled metabolites on alumina and a profile of radio-activity found in fractions after chromatography of an incubating medium on alumina.

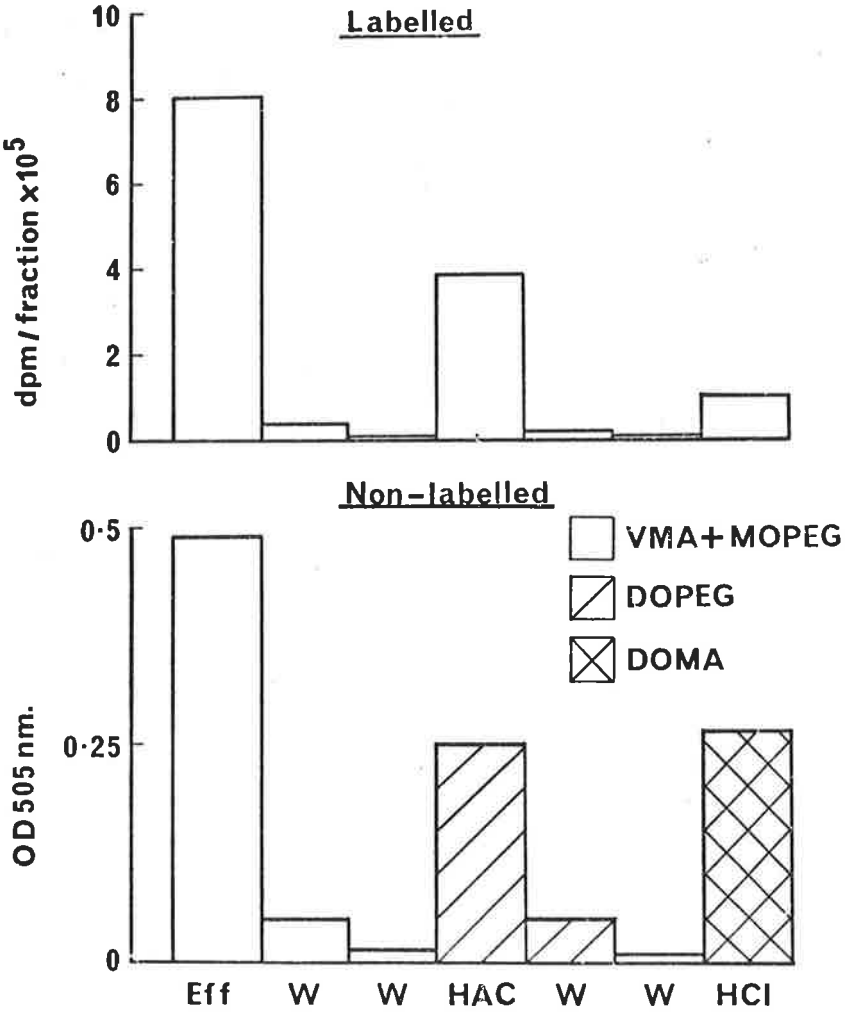

Fig. 3.4 Fractions obtained by alumina chromatography. Upper panel: Profile of radioactivity after chromatography of effluent from Dowex 50 columns. Lower panel: profile of unlabelled metabolites of NA. Unlabelled metabolites were applied separately to the alumina and detected in the different fractions by the colorimetric assay described in 3.5.

(b) Thin Layer Chromatography

A TLC method was developed which utilized the principle that catechols and not their 3-O-methylated derivatives are chelated by disodium tetraborate.

(i) Preparation of solutions for chromatography

Krebs incubation media were acidified at the end of the incubation period with HCl (100 mmol l^{-1}) and applied directly to TLC plates with a known amount (0.2 μmol) of NA and its metabolites. The unlabelled compounds were used for estimating the recoveries of tritiated NA and metabolites. One ml of the tissue extracts (1 mol l^{-1} acetic acid) was used to determine the total ^{3}H content of the extract. The remaining 2.5 ml was added to lypholization vials with 0.5 μmol of NA and each metabolite for estimating recoveries. Each sample was snap frozen with acetone-dry ice. After lyphilization each sample was immediately reconstituted in 0.5 ml of acetic acid (0.1 mol l^{-1}) in ethanol. 0.1 ml of this solution was spotted onto TLC plates. Tissues were extracted with acetic acid and reconstituted after lypholization with acetic acid in ethanol since the use of HCl at either of these stages resulted in the formation of extra fractions which did not co-chromatograph with any of the non-lyphilized catecholamines or 3-O-methylated derivatives.

(ii) Preparation of plates

Thin layer silica gel plates (Merck) containing fluorescent indicator were impregnated with disodium tetraborate (0.5 mol l^{-1}, pH 8.5) for a distance of 12 cms. from the origin and oven dried.

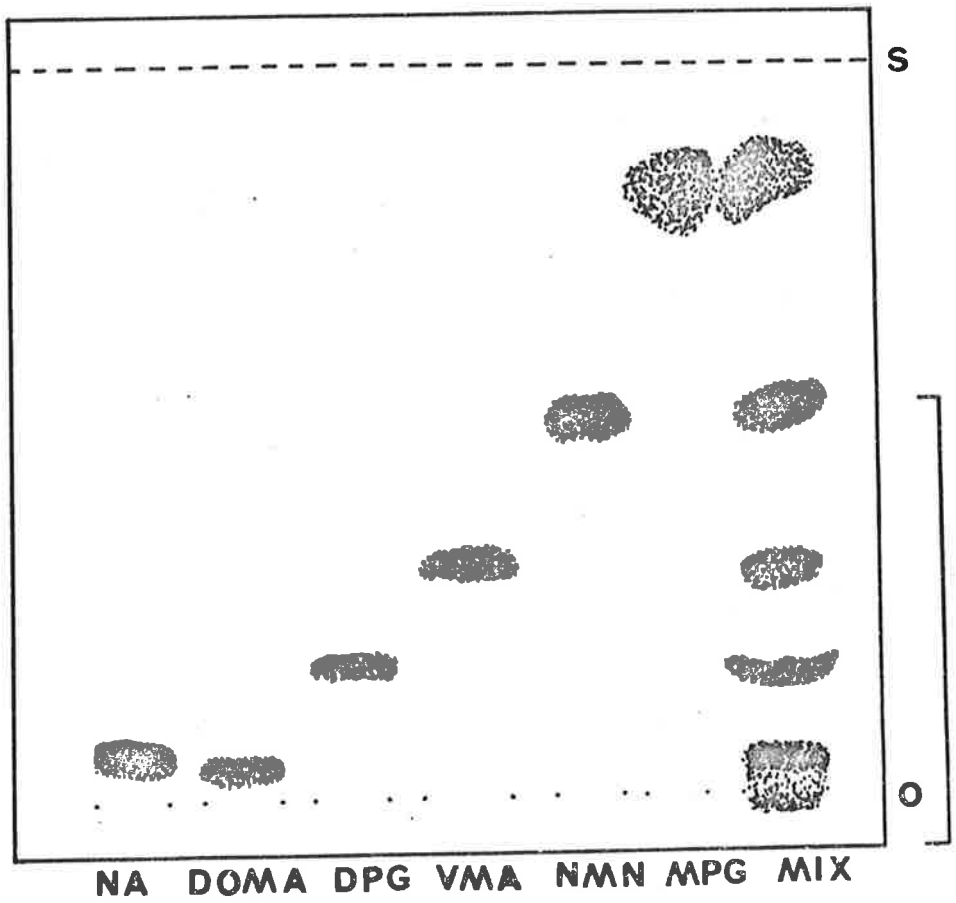

Fig. 3.5 Borate impregnated thin-layer chromatograms showing the separation of 3-O-methyl metabolites from NA and its catechol metabolites. O indicates the origin, S the solvent front. The area of borate impregnation is indicated by the vertical solid bar. Plates were sprayed with $K_3Fe_2(CN)_6/FeCl_3$.

(iii) Details of method

0.04 ml of incubating media and 0.1 ml of reconstituted tissue extracts containing unlabelled NA and metabolites were applied to the origins and the plates were developed in the solvent n-butanol: ethanol: Tris buffer (Sigma 7-9, 10 mmol l^{-1}, pH 8) in a ratio of 2:1:1 for 13 hours at room temperature. The 3-O-methylated metabolites migrated with the solvent and separated from each other on the plate. At pH 8.5, DOPEG also migrated to a limited extent within the borate impregnated area and separated from NA and DOMA which remained at the origin (fig. 3.5). The areas of the plate corresponding to the different metabolites could be visualized under U.V. light. These spots were scraped into tubes containing 2 ml of HCl (300 mmol l^{-1}) and eluted for 20 hours at $4^{o}C$. 1.0 ml of each eluate was used to estimate the ^{3}H content by liquid scintillation spectrometry and 1.0 ml was used to assay the amount of metabolites recovered by the method described in section 3.5. A profile of the radioactivity recovered from these plates is shown in fig. 3.6.

The amount of ^{3}H and the amount of unlabelled NA and metabolites recovered from these plates is shown in table 3.2. Since the major proportion of ^{3}H was found at the origin, the amount of NA lost accounts to a large extent for the ^{3}H not recovered.

(iv) Notes and Discussion

The major disadvantage of this method is that NA and DOMA are not separated from one another. However, the TLC method was

TABLE 3.2 Recovery of Tritium and unlabelled NA and metabolites after Thin Layer Chromatography.

	INCUBATION MEDIA		TISSUE EXTRACTS
	^{3}H lNA incubated with tissue	^{3}H lNA incubated without tissue	
% Recovery of ^{3}H.	80.1 ± 0.9 (n = 27)	82.7 ± 2.7 (n = 9)	62.5 ± 2.6 (n = 36)
% Recovery of Unlabelled Compounds			
NA	78.6 ± 2.3 (n = 11)		68.3 ± 2.9 (n = 20)
DOPEG	77.8 ± 2.1 (n = 71)		67.0 ± 1.8 (n = 75)
VMA	39.5 ± 1.9 (n = 71)		42.8 ± 1.6 (n = 76)
NMN	82.6 ± 1.6 (n = 73)		84.6 ± 1.8 (n = 76)
MOPEG	75.7 ± 1.5 (n = 73)		80.6 ± 1.8 (n = 76)

Footnote:

Recovery of Unlabelled compounds was estimated colourimetrically by the method described in Section 3.5.

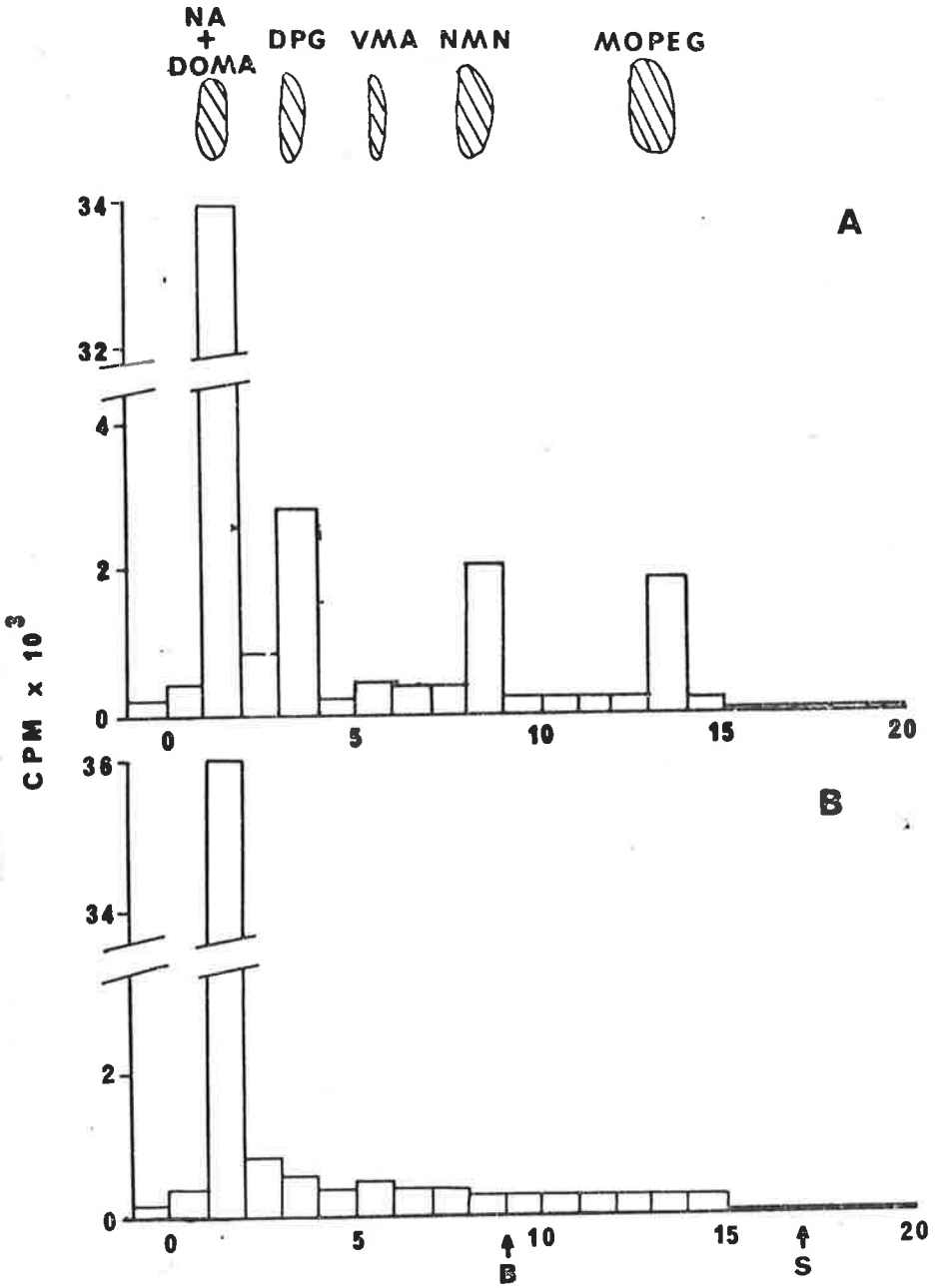

Fig. 3.6 The distribution of radioactivity on borate impregnated thin-layer chromatograms of samples of A: ^{3}H l NA incubated with uterine tissue and B: ^{3}H l NA incubated without tissue. The arrows indicate the position of the solvent front (S) and the 'borate front' (B). The migration of NA and its metabolites are shown.

TABLE 3.3 A comparison of the two separative techniques employed for NA and its metabolites with respect to the % cross contamination of each fraction by ^{3}H INA.

FRACTION	% CONTAMINATION	
	INCUBATION MEDIA	TISSUE EXTRACTS
D_{50}/Alumina		
VMA + MOPEG	4.56 ± 1.14 (n = 6)	3.85 ± 1.10 (n = 6)
DOPEG	2.50 ± 0.50 (n = 6)	2.30 ± 0.50 (n = 6)
DOMA	1.21 ± 0.12 (n = 6)	1.20 ± 0.12 (n = 6)
NMN	3.59 ± 0.90 (n = 6)	3.41 ± 0.80 (n = 6)
TLC		
DOPEG	1.27 ± 0.25 (n = 17)	2.31 ± 0.79 (n = 11)
VMA	0.63 ± 0.90 (n = 20)	1.16 ± 0.48 (n = 11)
NMN	0.23 ± 0.05 (n = 15)	0.51 ± 0.19 (n = 10)
MOPEG	0.11 ± 0.11 (n = 15)	0.17 ± 0.05 (n = 11)

Footnote: Cross contamination was estimated by the ^{3}H appearing in each metabolite fraction when pure ^{3}H INA was chromatographed.

used in preference to the D_{50}/Alumina procedure since the degree of contamination of metabolite fractions by ^{3}H NA was smaller and more consistent. This was particularly relevant when examining incubating media in which the metabolites represented a small fraction of the total ^{3}H present when compared to NA. The data in table 3.3 indicates that contamination of the o-methylated metabolite fractions by ^{3}H NA is very much lower using TLC. Although DOPEG has the highest % contamination of any fraction by TLC separation, it is no greater than the contamination found with D_{50}/Alumina separation. DOPEG should be estimated with a similar degree of precision by both methods while the o-methylated metabolites should be estimated more precisely by TLC.

Furthermore the TLC method was more rapid since a smaller number of fractions had to be counted and assayed per sample.

5. AUTOMATED COLORIMETRIC ASSAY FOR NORADRENALINE AND METABOLITES

The chromatography procedures used to separate ^{3}H NA and its metabolites resulted in some loss of these compounds. To correct for these losses, known quantities of unlabelled NA and its metabolites were added to the original test samples and their recoveries estimated after chromatography. These recovery values were then used to correct for the recovery of the corresponding tritiated compounds. The usual method of assaying these compounds is to measure the native fluorescence of phenolic compounds. This method was found to be unsuitable since high blank values were

obtained especially when assaying extracts of TLC plates since these contained a fluorescent indicator. A colorimetric assay was used which adapted the paranitroaniline method of visualizing catecholamines and their 3-O-methylated derivatives on chromatography media to its use on the technicon autoanalyser.

The samples and standard solutions of NA and metabolites were introduced into the manifold of the autoanalyser (fig. 3.7) at a rate of 40/hour. The samples (in 300 mmol 1^{-1} and 500 mmol 1^{-1} HCl) were made alkaline by the addition of potassium carbonate (1.5 mol 1^{-1}) and the diazonium derivatives were formed after the addition of freshly mixed para nitro aniline (7.2 mmol 1^{-1}) and sodium nitrite (29 mmol 1^{-1}). The resulting colored derivatives were passed through the flow cuvette of the colorimeter and the change in optical density at 505 nmetres recorded. A plot of OD_{505} against the concentration of NA and its metabolites is shown in fig. 3.8. The relationship was linear in the range 0 to 150 µmol 1^{-1} for all metabolites except DOMA and the change in absorbance was greater for 3-O-methylated compounds than for the catechols.

Some of the compounds commonly used as anti-oxidants in chromatographic procedures for catecholamines were tested for interference. EDTA and sodium metabisulphite contributed minimally to the change in optical density and their interference was only significant when they were present in solutions at concentrations greater than 5 and 0.5 mmol 1^{-1} respectively. The commonly used anti-oxidant, ascorbic acid, produced considerable interference and on a molar basis was

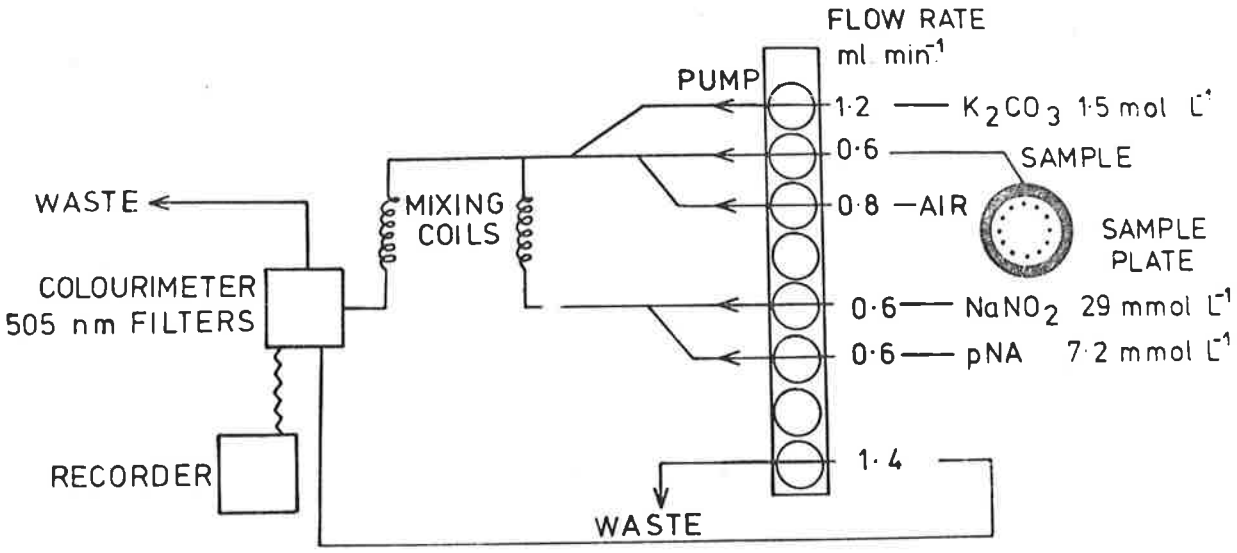

Fig. 3.7 A schematic representation of the automated colorimetric assay procedure for the measurement of catecholamines and their metabolites.

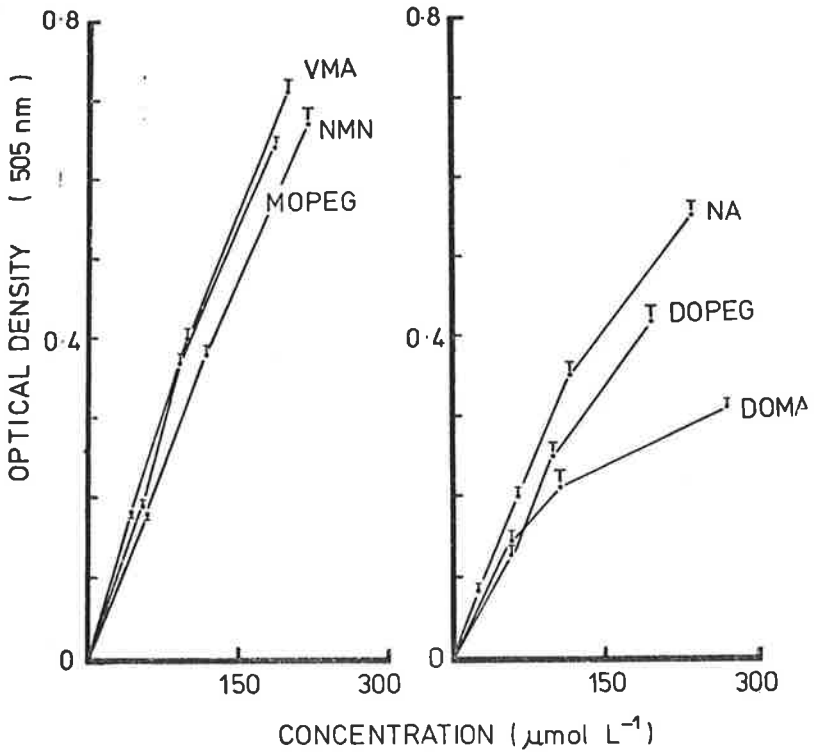

Fig. 3.8 Calibration curves for standard solutions of NA and its metabolites obtained using the automatic assay outlined in fig. 3.7. Abscissa: concentration of NA and its metabolites, ordinate: optical density at 505 nm. Values are means ± S.Es. for 12 or more experiments.

about one third as effective as NMN in producing a change in OD. Therefore sodium metabisulphite was used as the anti-oxidant in the chromatographic procedures described above. It was also found that the Tris buffer used for the solvent in the TLC separations reacted with this assay. When present in the solvent at concentration greater than 50 mmol 1^{-1} it produced a significant change in OD for extracts taken from the origin of TLC plates but not for extracts taken from elsewhere on the plates. The concentration of Tris buffer used in the solvent was therefore kept at 10 mmol 1^{-1}, in which concentration it did not contribute to the change in absorbance at 505 nm.

6. NOTES ON METHOD

Use of *in vitro* uterine segments

This section describes the characteristics of uterine segments used in the study of the *in vitro* accumulation and metabolism of NA.

(a) Region of Uterus Used

With the exception of the utero-tubal junction (Brundin, 1965) the sympathetic nerve terminals in the rabbit uterus are distributed uniformly along the length of the uterus from its junction with the oviduct to the cervix (Sjoberg, 1968). The utero-tubal junction is characterized by a band of circular smooth muscle which has a dense sympathetic innervation and is thought to function, along with the isthmus, as a sphincter (Brundin, 1965). Due to this uniform distribution of nerve terminals along the length of the uterus it

might be expected that the pattern of uptake of catecholamines would not vary along the length of the uterus. This was tested by comparing the accumulation of ^{3}Hl NA, an amine which is taken up by both the neuronal and extraneuronal system, and ^{3}Hdl isoprenaline which is mainly taken up by the extraneuronal system, in segments from different regions of the rabbit uterus.

The segments were incubated for 30 mins at 37^{o}C with ^{3}H l NA (1.2 µmol l^{-1}) or ^{3}H dl iso (1.2 µmol l^{-1}) followed by a 1.0 min wash in amine free Krebs solution to remove incubating medium adhering to the outside of the tissue. The results shown in table 3.4 indicated that there was a significantly greater uptake of NA than ISO, at a similar substrate concentration, implying that the uterus possessed an active neuronal uptake system for NA. However there was no significant difference between the ^{3}H content of segments from the proximal, middle and distal portions of the uterus indicating that the activities of both the neuronal and extraneuronal systems did not differ in the different regions of the uterus. In the experiments described in this thesis, segments were always prepared routinely from the middle region of each uterine horn, since although the uptake of NA was not different, NA metabolism may have varied with the region of uterus used.

(b) Dimensions of Uterine segments

As in any study on tissues *in vitro*, it was important that the tissue size be sufficiently small to permit rapid diffusion of drugs or nutrients to their sites of action or sequestration. Catan *et al.* (1968) estimated the limiting thickness of human uterine strips for O_2

TABLE 3.4 Accumulation of NA and ISO by different regions of the rabbit uterus

Position of segment relative to the oviduct	TOTAL ^3H nmol (g dry wt)$^{-1}$ (30 min)$^{-1}$	
	^3H l NA	^3H dl ISO
PROXIMAL	17.9 ± 1.2	7.13 ± 0.55
MID. SECTION	16.0 ± 1.9	6.56 ± 0.16
DISTAL	18.8 ± 3.6	7.58 ± 0.96

n = 4

consumption to be 1.5 mm when 100% O_2 was used and 1.1 mm when 50% O_2 was used for the gas phase. The limiting thickness for the uptake of catecholamines by rabbit uterine slices has not been determined. However, Graefe (1976) maintains that the access of the catecholamines to the uptake system is probably not limited by diffusion, if the uptake increases linearly with the size of the tissue slice. In recent studies Graefe (1977) has employed tissue slices 0.5 mm thick, however he does not provide data for uterine tissue. Most of the studies of catecholamine uptake by uterine tissue have been performed *in vivo* (Wurtman *et al.*, 1964). However, Green & Miller (1966a) investigated the uptake of NA and A in rat uterine slices. They used longitudinal slices of uterine tissue 0.26 mm thick from mature rats and unsliced uteri from immature rats. Using these preparations Green & Miller were able to demonstrate active uptake of NA but were unable to demonstrate metabolism of NA. Green & Miller do not provide comparative data for uterine slices of different thickness.

In order to choose rabbit uterine segments sufficiently small to allow ample diffusion but sufficiently large to produce measurable quantities of metabolites, preliminary experiments on segments of different sizes were necessary.

Since the sympathetic nerves are distributed throughout the mass of smooth muscle in the uterus, it would be expected that neuronal accumulation and oxidative deamination of NA would be

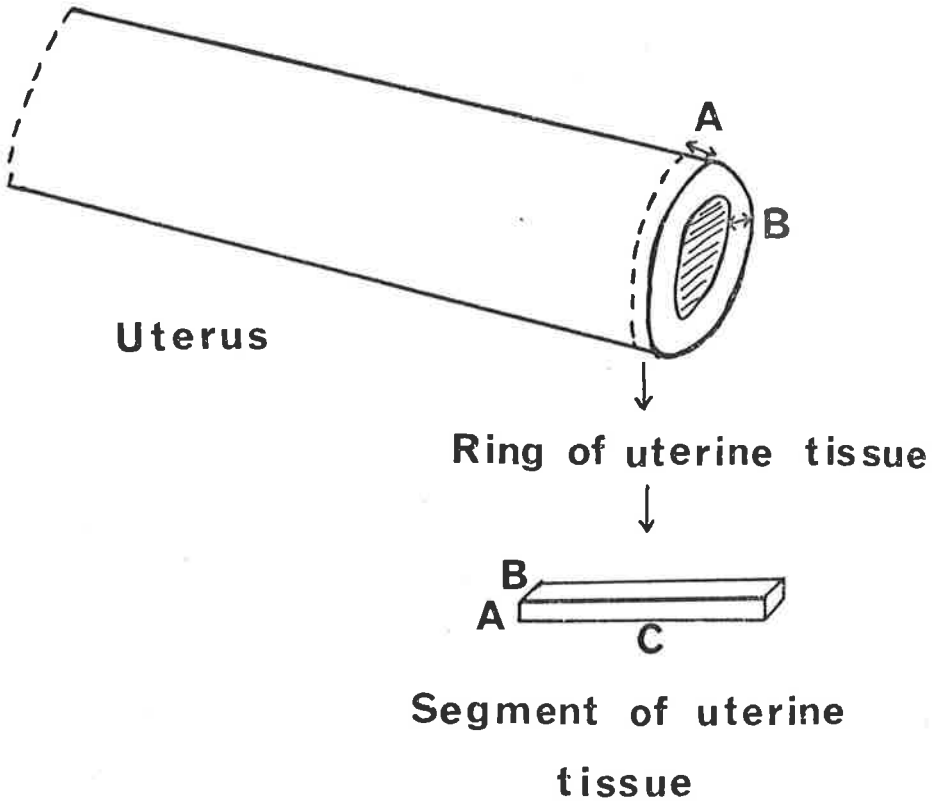

Fig. 3.9 Dimensions of uterine segments used for incubation studies. A section of uterus of length A was removed. The ring of tissue thus formed was cut open to form a segment of which A = the thickness; B = the thickness of the uterine wall; C = outer circumference of the uterus.

impeded by inadequate diffusion of NA and O_2 into segments. There is increasing evidence in a number of sympathetically innervated tissues that DOPEG, an oxidized product of NA, is the main metabolite produced intraneuronally (Levin, 1974 Graefe *et al.*, 1973). NA accumulation and metabolism to DOPEG was therefore investigated in uterine segments of different sizes.

(i) Effect of Segment Size on the Accumulation of ^{3}H l NA

The accumulation of ^{3}H l-NA per gram of tissue in uterine segments of different weights is shown in table 3.5. These segments were prepared from rings of tissue of different thicknesses, the thickness being equivalent to the length (A) of uterine section used (see Fig. 3.9). The 40 mg segments were 1.0 mm thick. All segments were prepared from the same uterine horn so that the thickness of the uterine wall (B) was as constant as possible. The segments were incubated with ^{3}H l NA as described in 3.6(a) above. The data in table 3.5 shows that the accumulation of ^{3}H per gram of uterus did not vary in segments from 18 to 42 mg wet weight. However tissue segments weighing 50 mg or greater accumulated less ^{3}H NA.

(ii) Effect of Segment Size on 3, 4 Dihydroxy phenyl glycol Production.

Uterine segments of different weights were prepared and incubated with ^{3}H l NA as described in i) above. The ^{3}H in the incubating medium and in the tissue extracts was analysed for DOPEG using TLC. Despite a weight range of 18.7 to 42.3 mg the data in table 3.6 indicates that the total DOPEG formation (tissue & medium content) shows little variation (less than 8%).

TABLE 3.5 Effect of size of uterine segments on accumulation of NA.

wet wt. (mg)	ACCUMULATION OF ^{3}H 1 NA	
	nmol (g dry wt)$^{-1}$((30 min)$^{-1}$	nmol (g wet wt)$^{-1}$ (30 min)$^{-1}$
18.7	15.83	1.69
18.8	14.90	1.74
21.4	17.88	2.00
29.4	15.99	1.69
32.2	16.50	1.74
41.2	15.34	1.64
42.3	15.22	1.66
50.0	10.76	1.25
51.7	10.23	1.23
54.9	9.14	1.26
71.4	9.88	1.19
88.2	9.06	1.01

40 mg segments correspond to 1.0 mm thickness

TABLE 3.6 Effect of segment size on DOPEG production

Wet wt. (mg)	DOPEG nmol (g dry wt)$^{-1}$ (30 min)$^{-1}$
18.7	4.44
21.4	4.95
29.4	4.17
32.2	4.89
41.2	4.28
42.3	4.08

mean DOPEG production = 4.47 ± 0.15 nmol (g dry wt)$^{-1}$ (30 min)$^{-1}$

Comment:

When incubated with ^{3}H l NA (1.2 μmol l^{-1}) for 30 min, the accumulated ^{3}H and the production of DOPEG was linearly related to the weight and thickness of uterine segments in the weight range 18 to 42 mg. Tissue segments of 50 mg or greater accumulated less ^{3}H per g of tissue so that in the experiments in this thesis, uterine segments were limited to 1.0 mm in thickness (length of uterine section) and 30-40 mg wet weight. An exception was necessary in the case of ovariectomized control animals described in Chapter 6. In some of these animals the uterine wall was very thin and in order to obtain 30-40 mg of tissue, sections of uterus longer than 1.0 mm were needed. Conversely in pregnant animals, uteri were very large. In these cases, segments were restricted to 1.0 mm in thickness and 40 mg weight necessitating the use of incomplete rings of tissue. Segments smaller than 30-40 mg were not used as it was thought that 30-40 mg of tissue would provide a better means of estimating the metabolites of NA which are produced in small quantities.

It should be noted that the uterine segments described here are composed of both endometrium and myometrium. No attempt was made to separate these two layers.

(c) Effect of Substrate Concentration

The accumulation of ^{3}H l NA and ^{3}H dl ISO by uterine segments was examined at substrate concentrations ranging from 0.1 μmol l^{-1} to 30 μmol l^{-1}. The segments were incubated for 30 min as described in 3.6(a) above. The results in table 3.7 indicate that ^{3}H l NA uptake was not saturable at the highest substrate concentration used

TABLE 3.7 Accumulation of ^{3}H l NA and ^{3}H dl ISO by rabbit uterus at different substrate concentrations.

Substrate Concentration mol l^{-1}	^{3}H l NA		^{3}H dl ISO
	^{3}H accumulated nmol (g dry wt)$^{-1}$ (30 min)$^{-1}$	$\frac{^{3}H \text{ tissue}}{^{3}H \text{ medium}}$	^{3}H accumulated nmol (g dry wt)$^{-1}$ (30 min)$^{-1}$
1.17×10^{-7}	2.21 ± 0.60	2.3	0.71 ± 0.12
1.18×10^{-6}	13.6 ± 1.14	1.4	8.3 ± 1.2
2.94×10^{-6}	27.2 ± 1.8	1.13	16.7 ± 2.5
5.88×10^{-6}	51.3 ± 1.7	1.06	35.0 ± 1.8
1.18×10^{-5}	87.6 ± 11.0	0.91	76.9 ± 7.7
1.77×10^{-5}	119.4 ± 8.0	0.83	134.0 ± 15.6
2.94×10^{-5}	202.4 ± 12.2	0.84	170.7 ± 14.6

1) n = 5, NA; n = 4, ISO

2) Tissue/medium ratio of ^{3}H was estimated using tissue values after 1 minute wash.

ie 29 μmol l^{-1}. Presumably this was due to the inability to saturate the extraneuronal uptake system which has been reported to have a high capacity for binding catecholamines (Iversen 1967, Graefe & Trendelenburg 1974). This is supported by the inability to saturate the accumulation of ISO at 29 μmol l^{-1}. By washing the tissue after incubation for 30 mins, thereby removing the more loosely-bound extraneuronal NA, the neuronal uptake system was found to be saturated at 5.9 μmol l^{-1} l NA. The substrate concentration employed for incubation studies in this thesis was 1.2 μmol l^{-1}. The actual tissue to medium ratio at 30 min incubation over the range of substrate concentrations could not be calculated since the tissues had been washed for 1 min in amine free Krebs solution before extraction. In chapter 5 it will be seen that approx. 10% of the total ^{3}H accumulated is lost from the tissue during this one minute wash period. The ratios given in table 3.7 are therefore an underestimate of the true tissue to medium ratios. Nevertheless, it was observed that the ratio of ^{3}H present in the tissue to that in the incubating medium declined with increasing substrate concentration. A similar decline in tissue to medium ratios of NA from 2 to 1.5 was observed by Levin (1974) for rabbit aorta over a concentration range 30 nmol l^{-1} to 3 μmol l^{-1}, a phenomenon he attributed to saturation of binding sites for NA in the tissue. In the case of the rabbit uterus it would appear that saturation applies predominantly to the neuronal system.

ADDENDUM

The concentration of ^{3}HNA (1.2 μmol l^{-1}) is less than the Km of the neuronal uptake system in rabbit heart (2.9 μmol l^{-1}) [Graefe, K-H. and Bönisch, H., 1978: "The influence of the rate of perfusion on the kinetics of neuronal uptake in the rabbit isolated heart." Arch. Pharmacol. 302: 275-283] and is also less than the Km of the extraneuronal catecholamine O-methylating system in the rat heart (1.7 μmol l^{-1}) [Fiebig, E.R. and Trendelenburg, U., 1978: "The kinetic constants for the extraneuronal uptake and metabolism of ^{3}H(-)-Noradrenaline in the perfused rat heart." Arch. Pharmacol. 303: 37-46.]

CHAPTER 4.

THE EFFECT OF OVARIAN STEROIDS ON THE NORADRENALINE CONTENT AND METABOLISM OF CATECHOLAMINES IN HOMOGENATE PREPARATIONS OF RABBIT UTERI

INTRODUCTION

Previous studies on catecholamines in the female reproductive tract of the rabbit have been concerned with changes in catecholamine content (Sjöberg, 1967, Falck *et al.*, 1969a & b, Brundin, 1965) and the characterization of adrenergic receptors (Marshall, 1970). Unlike the rat and human, no published data is available on the activity of the two metabolizing enzymes, MAO and COMT, in rabbit uterus.

The experiments described in this chapter were designed to determine the activity of MAO and COMT in homogenate preparations of rabbit uterus. The effect of pregnancy and the two hormones, 17β ostradiol and progesterone, on the activity of these two enzymes was then investigated.

The catecholamine content of the uterus was estimated for all treatments investigated as one index of the density of sympathetic innervation. The endogenous NA content of the rabbit uterus has been reported to alter under these treatment conditions (Sjöberg, 1967). The regime of Sjöberg (1967) and Falck *et al.* (1969a & b) for 17β ostradiol and progesterone pretreatment was employed in order to explore a possible association between NA content and MAO and COMT activity.

METHODS

The experimental animals were sexually mature female rabbits of mean body weight 2.84 ± 0.13 Kg. In experiments in which rabbits were pretreated with steroids, they were ovariectomized and allowed 4 weeks to recover from surgery. They were then divided into 4 groups which received the following treatment -

1) vehicle control animals: 0.1 ml/Kg of peanut oil containing ethyl alcohol (1.7 μmol l^{-1}) subcutaneously, daily for 14 days.
2) 17β oestradiol, 0.5 μg/Kg, subcutaneously daily for 14 days.
3) vehicle, subcutaneously, daily for 7 days followed by progesterone, 2 mg/Kg subcutaneously, daily for a further 7 days.
4) 17β oestradiol, 0.5 μg/Kg, daily for 14 days with progesterone, 2 mg/Kg, for the second 7 days of treatment.

Pregnant animals were studied at 28 days of pregnancy, day 0 being the day of mating.

The rabbits were bled from the marginal ear vein prior to killing. Plasma samples were frozen prior to assay for 17β oestradiol and progesterone content.

Progesterone was assayed by competitive protein binding to C.B.G. by the method of Murphy (1970). 17β oestradiol was assayed by the radioimmunoassay described by Janson *et al.* (1978).

One uterine horn was homogenized in phosphate buffer and assayed for MAO and COMT activity as described in Chapter 2. The endogenous NA and A content of the second uterine horn was assayed fluorimetrically as described in Chapter 3.

RESULTS

1. CATECHOLAMINE CONTENT

The effect of the various treatments on the size of the rabbit uterus is summarized in table 4.1. Ovariectomy caused a fall in the mean weight of the uterus from 1.66 g per uterine horn to 1.01 g. 17β oestradiol increased the weight of the uterus approximately two fold over the size of the uterus from ovariectomized control rabbits. Although the mean weight of the uterus increased after progesterone treatment this was not significantly different from control animals. When both steroids were given together there was a greater increase than when either steroid was given alone viz 3.7 fold. At 28 days after mating, in the pregnant rabbit, there was a large increase in the weight of the uterus, being approximately 12 times that of untreated animals. The ratio of dry to wet weight was similar for all treatments but these were significantly different from the uteri from untreated animals. The latter displayed a smaller degree of hydration having a significantly greater dry to wet weight ratio ($P < 0.001$, unpaired 2-tailed t-test).

The changes in NA concentration of uteri are presented in table 4.2, and the changes in uterine weight are relevant to the interpretation of the changes in NA concentration. When NA contents were expressed on a weight basis, be it dry or wet weight, they varied over a wide range, as indicated by the SEs, within a given group. However this variability was not evident when NA content was expressed per uterine horn. Ovariectomized control animals had 2.29 ± 0.12 nmol (0.39 ± 0.022 μg) of NA/uterine horn while untreated animals had 2.53 ± 0.24 nmol (0.43 ± 0.04 μg) of NA/uterine horn. The

TABLE 4.1 Effect of steroid treatment on uterine weight.

TREATMENT	WET WEIGHT (g)	DRY WEIGHT (mg)	DRY WEIGHT / WET WEIGHT
Untreated (n = 6)	1.66 ± 0.29	208 ± 38	0.13 ± 0.01
Ovariectomized control (n = 11)	1.01 ± 0.28	90.9 ± 25.2	0.09 ± 0.01
17β oestradiol (n = 8)	2.13 ± 0.19	192 ± 17	0.09 ± 0.01
Progesterone (n = 4)	1.30 ± 0.55	117 ± 50	0.09 ± 0.01
17β oestradiol + progesterone (n = 4)	3.69 ± 0.44	310 ± 37	0.08 ± 0.01
Pregnant (n = 5)	19.5 ± 0.7	1402 ± 50	0.07 ± 0.01

Footnote: All weights refer to the weight of one uterine horn, total uterine weight being approximately double the values shown.

variability of NA content when expressed on a weight basis appeared to be due to the variability in uterine weight ranging from 0.08 to 3 g/uterine horn for ovariectomized control animals and 0.3 to 2.4 g for untreated animals. Fig. 4.1 depicts the relationship between uterine weight and NA content in nmol (g wet weight)$^{-1}$ for ovariectomized control rabbits. This relationship can be described by an exponential equation derived from the observed points of $y = 1.99\ e^{-1.17x}$. When the corresponding values for untreated rabbit uteri were plotted in the same way, they were found to lie on the same line as that for ovariectomized control rabbits. Moreover from the data in table 4.2, it can be seen that, although the NA content of ovariectomized and untreated animals differed when expressed on a weight basis, the total amount of NA per uterine horn was not significantly different.

With the exception of progesterone treatment alone, the steroid treatments and pregnancy caused significant increases in uterine weight and decreases in the content of NA expressed on a weight basis when compared to ovariectomized control animals. However the amount of NA per uterine horn was not significantly different for any of the uteri from these steroid treatment groups when tested by an unpaired t test. Although there was a tendency for NA content per uterine horn to increase after 17β oestradiol treatment, this did not reach significance ($0.05 < p < 0.10$). In fig. 4.1 it can be seen that only two of the eight animals treated with 17β oestradiol had uterine NA contents in nmol (g wet wt.)$^{-1}$ substantially above the line describing the ovariectomized control animals.

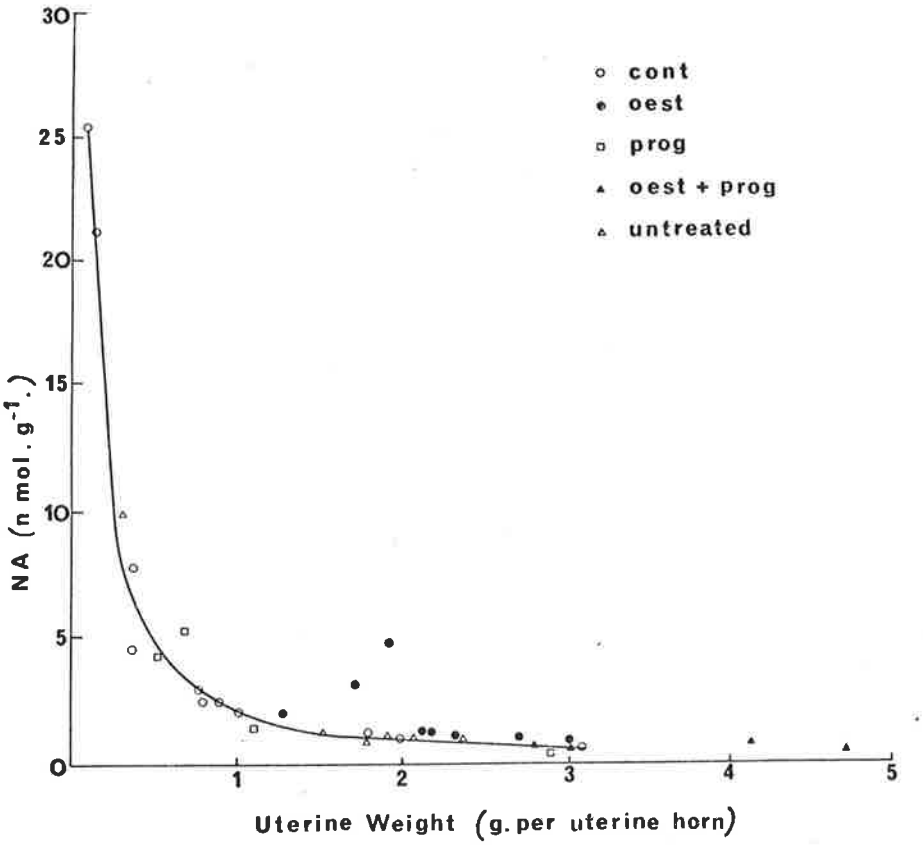

Fig. 4.1 Relationship between endogenous NA concentration and organ weight in the rabbit uterus. The exponential curve has been drawn for the points plotted for ovariectomized control animals.

TABLE 4.2. The effect of steroid treatment on the endogenous catecholamine content of rabbit uterus.

TREATMENT	NA CONTENT			A CONTENT
	nmol/g wet wt.	nmol/g dry wt.	nmol/ uterine horn	nmol/ uterine horn
Untreated (n = 6)	2.88 ± 1.65	23.8 ± 13.9	2.53 ± 0.24	0.10 ± 0.04
Ovariectomized control (n = 11)	7.35 ± 2.94	97.7 ± 46.5	2.29 ± 0.12	ND
17β oestradiol (n = 8)	2.06 ± 0.59	21.8 ± 4.8	3.94 ± 1.00	ND
Progesterone (n = 4)	3.29 ± 1.41	36.6 ± 20.9	2.47 ± 0.71	ND
17β oestradiol & progesterone (n = 4)	0.71 ± 0.06	8.47 ± 0.35	2.65 ± 0.35	ND
Pregnant (n = 5)	0.12 ± 0.06	1.82 ± 0.53	2.53 ± 0.71	0.03 ± 0.02

Footnote: 1) ND = not detectable.

2) Values are means ± S.E.Ms.

3) Ovariectomized control v. 17β oestradiol

nmol NA/uterine horn

$0.05 < P < 0.10$ (unpaired 2-tailed t-test)

The concentration of NA in pregnant uteri was very low, being 0.12 nmol (g wet wt.)$^{-1}$ (0.02 μg g^{-1}) or approximately 4% of that found in untreated animals. This effect also appeared to be due to changes in uterine weight since the total amount of NA per uterine horn was the same as for untreated and ovariectomized control animals.

The adrenaline content of the uterus was very low, being 0.10 ± 0.04 nmol (uterine horn)$^{-1}$ (0.017 ± 0.007 μg) in untreated animals. Adrenaline was not detectable in uteri from ovariectomized control animals or after steroid treatment. In 28 day pregnant rabbits the amount of A measured was 0.03 ± 0.02 nmol (uterine horn)$^{-1}$ (0.005 ± 0.003 μg).

In table 4.3 the NA and A contents of the rabbit ovary and oviduct are compared to those of the uterus. The oviduct has the largest NA content on a weight basis being approximately double that of the uterus while the ovary displayed the lowest NA content per g of tissue. The oviduct also contained approximately 25% as much A as NA while no A was detectable in the ovary.

2. ENZYME ACTIVITIES

The activities of MAO and COMT in uterine homogenates is shown in table 4.4. MAO activity (513 ± 100 nmol (mg protein)$^{-1}$ hr^{-1}) was much higher and more variable than COMT activity (1.34 ± 0.06 nmol (mg protein)$^{-1}$ hr^{-1}). There was no significant change in MAO or COMT activity from these values after bilateral ovariectomy.

TABLE 4.3 Endogenous catecholamine content of three regions of the female reproductive tract in untreated rabbits.

	UTERUS	OVIDUCT	OVARY
NORADRENALINE			
nmol/g wet wt.	2.88 ± 1.66	4.88 ± 0.85	1.76 ± 0.52
nmol/g dry wt.	23.8 ± 13.9	17.0 ± 2.6	9.50 ± 1.48
nmol/organ	5.06 ± 0.47	0.76 ± 0.08	0.92 ± 0.52
ADRENALINE			
nmol/g wet wt.	0.04 ± 0.02	1.19 ± 0.22	ND
nmol/g dry wt.	0.41 ± 0.22	4.03 ± 0.70	ND
nmol/organ	0.18 ± 0.08	0.18 ± 0.02	ND

Footnote: ND = not detectable

n = 6

(a) MAO

MAO activity was significantly increased in uteri from rabbits treated for 14 days with 17β oestradiol, 0.5 μg/Kg, when compared with ovariectomized control animals ($p < 0.001$, 2-tailed unpaired t-test). Progesterone, 2 mg/Kg, for 7 days also significantly increased MAO activity ($p < 0.05$). In the group which received both steroids, MAO was significantly increased above the control value ($p < 0.05$) although not to as high a level as in those animals given either steroid alone.

Pregnancy, at 28 days after mating, caused no significant change in uterine MAO activity when compared to untreated rabbits. However there was a significant increase in pregnant rabbit uterine MAO when compared to ovariectomized controls ($p < 0.05$).

MAO activity was not estimated on a tissue weight basis. However, since the protein concentration of mitochondrial preparations from the different treatment groups were all similar, the same results would be expected if enzyme activity was expressed per g of tissue. If one were to compare the deaminating capacity of the whole uterus then the effect of the steroids and pregnancy would be exagerated since all of these treatments (except progesterone alone) increased uterine weight.

(b) COMT

17β oestradiol significantly increased uterine COMT activity, when given alone ($p < 0.02$) or when given in combination with progesterone ($p < 0.02$). Progesterone when given alone for 7 days at 2 mg/Kg daily had no effect on COMT activity. There was no

TABLE 4.4 Effect of ovarian steroids on the activity of MAO and COMT in rabbit uterine homogenates.

TREATMENT	ENZYME ACTIVITIES nmol product (mg protein)$^{-1}$ hr^{-1}	
	MAO	COMT
Untreated (n = 10)	513 ± 100	1.34 ± 0.06
Ovariectomized control (n = 5)	429 ± 54	1.32 ± 0.04
17β oestradiol (n = 5)	1839 ± 136***	2.26 ± 0.29**
Progesterone (n = 5)	917 ± 98*	1.41 ± 0.33
17β oestradiol & progesterone (n = 5)	712 ± 64*	2.57 ± 0.37**
Pregnant (n = 5)	743 ± 115*	1.47 ± 0.25

Footnotes: 1) Values are means ± SEs.

2) MAO assay : substrate = ^{3}H tyranine

COMT assay: substrate = dihydroxy benzoic acid

methyl donor = ^{14}C S adenosyl methionine

3) * $P < 0.05$

** $P < 0.02$

*** $P < 0.001$

for unpaired, 2 tailed t test.

significant effect of pregnancy, at 28 days after mating, on uterine COMT activity when compared with either untreated rabbits or ovariectomized control animals. The total o-methylating capacity of the uterus, however, would be greatly increased in the pregnant rabbits since uterine weight increased greatly, the increase in weight of the tissue being paralleled by an increase in protein content. Similarly, the effect of 17β oestradiol would be exagerated if one considered its effect on the o-methylating capacity of the whole uterus whereas progesterone, which did not significantly increase uterine weight, would be without effect.

The data in table 4.5 indicates that MAO and COMT activity of liver homogenates greatly exceeded that of uterine homogenates except for the 17β oestradiol pretreated animals. The MAO and COMT activity of the rabbit liver was not affected by the steroid treatments or by pregnancy at 28 days after mating. Therefore, at the doses of 17β oestradiol and progesterone used, uterine MAO and COMT were more sensitive to these steroids than the corresponding liver enzymes. The plasma concentration of progesterone at the time of sampling was 9.56 ± 1.57 ng. ml^{-1} while 17β oestradiol was less than 20 pg. ml^{-1}, the lower limit of sensitivity of the assay.

TABLE 4.5 Effect of steroid pretreatment on rabbit liver enzyme activity.

	LIVER ENZYME ACTIVITY nmol product (mg protein)$^{-1}$ hr^{-1}	
	MAO	COMT
Control (ovariect.)	2035 ± 235	2.56 ± 0.34
17β oestradiol	1892 ± 342	2.41 ± 0.26
Progesterone	1966 ± 200	2.92 ± 0.55
17β oestradiol & progesterone	2004 ± 296	2.82 ± 0.42
Pregnant	2093 ± 250	2.58 ± 0.35

Footnotes: n = 5 for all treatments.

DISCUSSION

1. UTERINE WEIGHT

In accord with its known hypertrophic and hyperplasic effects on the rabbit uterus (Koseki & Fujimoto 1974), 17β oestradiol increased uterine weight, two fold in the dose used. Although progesterone did not significantly increase uterine weight when given alone, it caused a further increase in uterine weight when given with 17β oestradiol to 3.7 fold over controls. An effect of progesterone when given alone may have been masked by the large SE of this group, which may have reflected the small number of observations (n = 4). However Koseki and Fujimoto (1974) demonstrated that the threshold dose of progesterone for increasing the weight of the unprimed uterus of New Zealand rabbits was 2 mg/Kg, the dose used in the present case. Their dose response curve was very steep so that it is likely that the dose used was subthreshold in the present study. Moreover the effect of progesterone may have been confined to the endometrium since Koseki and Fujimoto (1974) demonstrated a selective effect of this dose of progesterone on endometrial hyperplasia as opposed to myometrial hyperplasia. Pregnancy caused a marked increase in uterine weight being approximately 12 fold at 28 days after mating. The ratio of dry to wet weight of the uterus was similar for all treatments except for untreated rabbits where uteri displayed the smallest degree of hydration of all groups. Nevertheless in subsequent chapters, the metabolites of NA are expressed on a dry weight basis to avoid the possibility of differences in the degree of hydration and any variability in the degree of blotting of wet tissues prior to weighing.

2. NA CONTENT

In untreated uteri, the NA content was 2.88 ± 1.65 nmol g^{-1} wet weight of tissue, which is low compared with other rabbit tissues such as the ear artery (19.5 ± 1.3 nmol g^{-1}). This implies that the rabbit uterus is less densely innervated than these tissues although it has not been directly tested in this study whether NA content is a measure of the density of sympathetic innervation in the uterus. The rabbit oviduct appears to have a denser sympathetic innervation than the uterus (4.88 ± 0.85 nmol g^{-1}) while the ovary appears to be less densely innervated (1.76 ± 0.52 nmol g^{-1}).

The adrenaline content of the rabbit uterus was found to be very low which agrees well with the findings of Sjöberg (1967) and Miller and Marshall (1965). The rabbit differs from the rat which has a relatively high uterine A content, much of this A being located at a non-vesicular site (Wurtman *et al.*, 1964). It might therefore be expected that quite significant differences in the uptake of catecholamines would be found between rat and rabbit uteri.

When related to either dry or wet weight of the uterus, ovariectomy caused an 150% increase in NA concentration. When compared to ovariectomized control animals, 17β oestradiol reduced NA concentration by 60% while progesterone alone was without effect. When the two steroids were given in combination there was a 75% decrease in NA concentration, while in uteri from pregnant animals NA concentration represented only 4% of that in untreated animals.

However, if uterine weights were taken into account, and the NA content expressed per uterine horn, none of these changes were significant. This implies that the extent of the sympathetic innervation to the uterus had not changed and the effects of the treatments on uterine weight were responsible for the apparent change in concentration of NA. The density of innervation tended to be greatest in the tissue showing the smallest weight (ovariectomized) and smallest in tissues showing the greatest increase in weight (progesterone + 17β oestradiol treated and pregnant rabbits).

The present results contrast with those of Sjöberg (1967) and Falck *et al.* (1969a,b) although they agree with those of Bell (1974) who demonstrated no change in NA content of the pregnant guinea pig uterus in spite of reduced catecholamine fluorescence. Miller and Marshall (1965) also failed to demonstrate an increase in NA content of the whole rabbit uterus after oestrogen treatment although they used a higher dose for a shorter time.

Falck *et al.* (1969b) used the same treatment regime as reported here and found a stimulating effect of 17β oestradiol on total NA content of rabbit uteri (table 4.6). Although total NA content showed a tendency to increase after 17β oestradiol in the present study this was due entirely to higher NA contents found in only two rabbits out of eight. Sjöberg (1967) also reported a decline in total NA content of the rabbit uterus after 21 days of pregnancy. In the present study no such decline was observed since the total NA content of the uterus at 28 days of pregnancy was the same as in untreated rabbits. The discrepancy between these two studies is unlikely to be due to the method of measuring NA content

TABLE 4.6 Data from Falck *et al.* (1969b) for rabbit uteri.

	UTERINE WT. (g)	µg/g NA	µg/PAIR OF UTERINE HORNS
Ovariectomized control	0.94 ± 0.34	0.77	0.73 ± 0.09
17β oestradiol	3.85 ± 0.37	0.41	1.59 ± 0.13
17β oestradiol & progesterone	6.00 ± 0.26	0.11	0.63 ± 0.11
Untreated (Sjöberg 1968)	0.80	0.90	0.70

since essentially similar techniques were employed. Moreover the total amount of NA per uterine horn from untreated rabbits in the present study (0.43 µg or approximately 0.8 µg/uterus) agrees well with the value of 0.73 µg/uterus reported by Falck *et al.* (1969b). The uterine weight in untreated rabbits in Sjöberg's (1967) study was much lower (0.80 g) than in rabbits used in the present study (3.2 g) so that the amount of NA per g of tissue in their studies was correspondingly much greater. However uterine weight after 17β oestradiol treatment was similar in the two studies. In the present study, rabbits were used when they had just reached sexual maturity. Although their body weights were in the weight range used by Falck *et al.* it is possible that these authors either used younger rabbits or that there is a difference in uterine weight between the two strains of rabbit at sexual maturity. The failure to demonstrate a significant increase in NA content of uteri after oestrogen treatment in the present experiment may reflect differences in the age or strain of the rabbits used or a difference in the dose of steroid required to elicit this effect. Hervonen *et al.* (1972) have demonstrated that 17β oestradiol increases the density of vesicles in the sympathetic nerve terminals of the rabbit uterus by EM techniques, using twice the dose of oestrogen employed by Falck *et al.*

The effect of oestrogen on NA content has also been reported by Falck *et al.* (1974) to explain the decline in NA content of the guinea pig uterus after bilateral ovariectomy. No such decline was observed in the rabbit uterus in the present study. However if one compares the data of Falck *et al.* (1969b) and Sjöberg (1968)

for rabbit uteri it is evident that they also failed to demonstrate a decline in total uterine NA content after ovariectomy in the rabbit.

3. ENZYME ACTIVITIES

MAO activity in the rabbit uterus greatly exceeded that of COMT. However both A and B iso-enzymes of MAO are estimated when tyramine is used as a substrate as in the present case. Only type A MAO has a high affinity for NA as measured in rat brain mitochondria and sympathetic ganglia (Goridis & Neff 1971). Attempts were made in the present study to measure type A MAO in the rabbit uterus but were unsuccessful. The two methods attempted are found in Appendix 2, one using radiolabelled NA as the substrate (Jain *et al.*, 1973) and one using clorgylene, a selective inhibitor of type A MAO, and ^{3}H tyramine as substrate (Goridis & Neff, 1971). Neither method detected measurable amounts of type A MAO in rabbit uterus which may mean that this isoenzyme is quite small in activity relative to type B in the rabbit uterus. However, this possibility needs further clarification since, using the same technique as Goridis & Neff, lower values of type A MAO were obtained for rat brain homogenates than those authors have reported (see appendix 2). This may mean that conditions were not ideal for detecting type A MAO in the present study.

Uterine MAO activity as measured using tyramine as a substrate was unaffected by ovariectomy. However all of the steroid treatments increased uterine MAO activity relative to the ovariectomized controls, the greatest increase (4 fold) being observed after oestrogen treatment. Pregnancy caused a significant increase

in uterine MAO activity when compared to ovariectomized controls but not when compared to untreated rabbits. This was perhaps surprising in view of the fact that both oestrogen and progesterone increase MAO activity and both steroids are elevated at this stage of pregnancy in the rabbit. However, it is not so surprising in light of the fact that, when both steroids were given concurrently the increase in uterine MAO activity was less than when either was given alone. The fact that uterine MAO activity was higher in pregnant animals than in ovariectomized controls but not untreated animals may reflect the reduced oestrogen production in ovariectomized animals.

COMT activity was increased approximately two fold by 17β-oestradiol pretreatment either alone or in combination with progesterone. Progesterone itself was without effect on COMT activity. Pregnancy also failed to alter uterine COMT activity.

The effects of steroid pretreatment on MAO and COMT activity would be qualitatively similar if one considered the enzyme activity of the whole uterus since all treatments except progesterone alone increased uterine weight. However, the o-methylating capacity of the uterus as a whole would be expected to increase with pregnancy since the weight and size of the uterus was greatly increased 28 days after mating. Similarly, the deaminating capacity of the whole uterus would have been increased by pregnancy.

At the doses of steroids used, the effects on MAO and COMT were relatively specific to the uterus in so far as liver MAO and COMT activity did not alter. This may, of course, reflect the relative degrees of binding of the two steroids by different tissues. One might expect a target tissue like the uterus to have a much higher tissue content of these two steroids than non target tissues. Specific receptors for oestrogen and progesterone have been described for the rabbit uterus by El-Banna & Sacher (1977). Unfortunately tissue levels of oestrogen and progesterone were not measured.

The stimulatory effect of progesterone on uterine MAO activity in the rabbit is similar to that reported for the rat and human. However there appears to be a species difference in the response to 17β oestradiol in that uterine MAO activity increased in the rabbit but has been shown to decrease in the rat and human (Southgate *et al.*, 1969, Holzbauer & Youdim 1973). The different response to 17β oestradiol in the present study does not appear to be due to the substrate used since Southgate (1972) reported similar effects on MAO activity assayed using a range of substrates including tyramine; dopamine was the only substrate used which gave different results. The competitive inhibition of rat uterine MAO by 17β oestradiol, demonstrated *in vitro* by Collins and Southgate (1970) contrasts with the stimulatory effect of 17β oestradiol pretreatment on rabbit uterine MAO. However, if such an inhibitory interaction occurs *in vivo* in the rabbit, it probably would not be detected in the mitochondrial preparations used here since the preparatory procedures would probably remove much of the steroid. However

Southgate *et al.* (1969) used similar mitochondrial preparations of rat uterus, so that differences between the two sets of results do not appear to be due to the enzyme preparation used.

Holzbauer & Youdim (1973) have suggested that increased MAO activity might result from increased permeability of the mitochondrial membrane or from the removal of a pre-existing inhibition. Specific enzyme synthesis was discounted due to the long half life of MAO compared with the rapid changes in MAO observed during the oestrous cycle in the rat. However it seems unlikely that changes in the permeability of the mitochondrial membrane is a factor since similar responses of rat uterine MAO to steroids have been demonstrated by Collins and Southgate (1970) using solubilized enzyme preparations.

Rabbit uterine COMT activity was stimulated by 17β oestradiol and unaffected by progesterone and pregnancy. Giles and Miller (1967) reported increased COMT activity in the rat uterus during oestrous compared with dioestrous, which might be related to the pro-oestrous surge of oestrogen production in the rat. However the literature is conflicting in that Salseduc *et al.* (1966) reported decreased activity of rat uterine COMT at oestrous compared with dioestrous. Neither author has reported the effects of exogenous steroids on uterine COMT activity, making comparison with the present results in the rabbit difficult.

The results from pregnant rabbit uteri differ from those in the rat in which uterine COMT activity has been reported to double during pregnancy (Wurtman *et al.*, 1964). In both species however, uterine MAO activity did not increase during pregnancy when compared with untreated animals.

Finally, it should be noted that there is no obvious association between MAO and COMT activities and NA content of the rabbit uterus. Thus, although combined oestrogen and progesterone treatment increased MAO and COMT activities of the uterus in association with a decrease in its NA concentration, a causal relationship seems excluded by the fact that oestrogen, alone, increased COMT activity to the same extent and MAO activity to a much greater extent and yet was associated with a much smaller decrease in NA concentration. The absence of a simple relationship between NA content and the activity of its metabolizing enzymes is not surprising in view of the many qualifying factors which may also operate. The effect of these treatments on the synthesis of NA in the rabbit uterus is not known. In rat superior cervical ganglia Hanbauer (1976) has shown that the activity of tyrosine hydroxylase, which catalyses the rate-limiting step in NA synthesis is altered by steroids such as dexamethazone. Moreover, in the intact tissue, the extent of the inactivation of NA will be determined by the ease of access of the amine to the sites of location of these enzymes. This last factor is one of the considerations in later chapters of this thesis.

CHAPTER 5

THE METABOLISM OF NORADRENALINE BY ISOLATED RABBIT UTERUS

INTRODUCTION

The purpose of the experiments described in this chapter was to quantitate the metabolites produced by isolated segments of uterus from untreated rabbits. In subsequent chapters, where the effect of ovarian steroids on these metabolites is assessed, control data were more appropriately obtained from ovariectomized animals. Data from ovariectomized animals are included therefore in this chapter for comparison with untreated animals.

In order to validate the metabolites identified in these experiments the effect of inhibition of COMT and MAO on the metabolite fractions was investigated. An inhibitor of neuronal uptake, cocaine, was used to indicate whether these metabolites were produced at a neuronal or extra-neuronal site. Additional information on the site of production of metabolites was sought by comparing the metabolism of lNA and dlNA since the l isomer is reported to be selectively accumulated by nerve cells (Iversen 1967).

The metabolites produced by the rabbit uterus were then compared with those from other reproductive tissues in the rabbit as well as from two other rabbit tissues which have been studied by similar techniques viz. the ear artery and aorta.

METHODS

Uterine segments, ovaries and oviducts were prepared and incubated with ^{3}H lNA (1.2 μmol l^{-1}) in Krebs solution at 37°C for 30 min. (as described in chapter 2). At the end of incubation the tissues were

washed with ^{3}H l NA-free Krebs solution for 10 secs and then for a further 50 secs before being placed in extraction solutions. The 10 sec wash solution was discarded. Metabolites were measured in the incubating medium and tissue extraction media and, in some instances, in the 50 sec wash solutions. Uterine metabolites were separated by either TLC or D_{50}/alumina chromatography in separate experiments (i.e. the same examples were not processed by the two separative techniques simultaneously). Metabolites from ovaries and oviducts were separated by TLC only.

The concentration of metabolites was expressed in terms of dry weight of tissue. After extraction of metabolites the tissues were dried over conc H_2SO_4 until the weights were constant. In all cases the wet weight was also determined.

Drug Treatments

Cocaine was dissolved in saline and U0521 in saline containing ascorbic acid (290 μmol l^{-1}) prior to their addition to Krebs incubating solutions in a volume of 0.01 ml/ml of Krebs solution. The tissues were exposed to either drug for 30 mins prior to their incubation with ^{3}H NA. They were then transferred to incubating solutions containing ^{3}H NA and either cocaine (29 μmol l^{-1}) or U0521 (55 μmol l^{-1}).

Nialamide was dissolved in HCl (1 mol l^{-1}) and immediately added to the Krebs solution whose pH was subsequently readjusted to 7.4. The tissue was incubated in this solution (350 μmol l^{-1} nialamide) for 1 hour and then further incubated for 15 min in nialamide-free Krebs solution prior to its incubation with ^{3}H NA.

RESULTS

1. ^{3}H ACCUMULATED

Table 5.1 summarizes the results obtained from isolated uterine segments from untreated rabbits incubated with ^{3}H l NA. The total ^{3}H accumulated by the tissue, obtained by summing the values for ^{3}H in the tissue after a 1.0 min wash and ^{3}H in the 50 sec wash solution was 2.1 + 0.27 nmol (g wet wt)$^{-1}$ (or 17.1 + 2.21 nmol (g dry wt)$^{-1}$, the ratio of wet to dry weights for untreated uteri being 8.20 ± 0.29 : n = 12). This value, 2.38 nmol (g wet wt)$^{-1}$, was significantly greater than the concentration in the medium (1.2 nmol ml^{-1} ^{3}H l NA) to indicate that the tissue had accumulated ^{3}H against a concentration gradient. However, the tissue content of unchanged ^{3}H l NA, 1.38 nmol (g wet wt)$^{-1}$ (30 min)$^{-1}$ was only 1.2 fold greater than the concentration in the medium and represents a poor accumulation of unchanged ^{3}H l NA by the tissue.

An estimate was also made of the amount of ^{3}H present in the extracellular space after a one minute wash. For this purpose segments were incubated with ^{14}C sorbitol (8.1 μmol l^{-1}) and unlabelled NA (1.2 μmol l^{-1}) under conditions identical to those for segments incubated with ^{3}H l NA. The ^{14}C sorbitol content after a 1 minute wash in amine and sorbitol-free Krebs was equivalent to 0.40 ± 0.01 nmol (g wet wt)$^{-1}$ (3.24 ± 0.06 nmol (g dry wt)$^{-1}$; n = 4) of ^{3}H l NA. Assuming that the ^{3}H l NA effluxed from the extracellular space at the same rate as ^{14}C sorbitol, the result suggests that no more than 20% of the ^{3}H material in the uterus was present in the extracellular space.

TABLE 5.1 Distribution of ^{3}H in tissue extracts and wash solutions after incubation of rabbit uterus with ^{3}HNA.

	^{3}H in tissue extracts		^{3}H in 50 sec wash solutions	
	A	B	A	B
NA	1.14	9.32	0.24	1.99
Metabolites of NA	0.67	5.52	0.03	0.22
Total ^{3}H	2.10	17.08	0.27	2.21

Footnote: (1) A refers to ^{3}H as nmol (g wet wt)$^{-1}$, (30 min)$^{-1}$

B refers to ^{3}H as nmol (g dry wt)$^{-1}$, (30 min)$^{-1}$

(2) Estimates were made in uteri from untreated rabbits.

2. DISTRIBUTION OF METABOLITES

The distribution of ^{3}H l NA and metabolites in tissues and incubating media was first examined by column chromatography, but this technique was later abandoned in favour of the more rapid and precise TLC method (see Chapter 3 for a comparison of the two methods) except when it was necessary to measure DOMA in occasional experiments. The early data for untreated uteri obtained by the column technique is compared to that obtained by TLC in table 5.2. The major discrepancy was a three fold greater NMN value when metabolites were separated by column chromatography. The VMA + MOPEG content of incubating media was also higher when estimated by column chromatography than by TLC. However it will be shown subsequently that the COMT inhibitor, UO521, only partially inhibited NMN formation when assayed by the column method but inhibited it by 87% when assayed by TLC. indicating the probability that the error lay with the former method. Except where otherwise specified, the following account refers only to data obtained by TLC.

(a) Untreated Uteri

(i) ^{3}HlNA

The data in table 5.2 indicate that the major proportion of the ^{3}H content of the tissue was ^{3}HlNA (54%) followed by VMA (10%) and DOPEG (8%). It should be noted that the sum of the individual fractions in the tissue represented 86% of the ^{3}H estimated from the tissue extract before chromatography.

The content of metabolites in the medium exceeded that in the tissue, the medium to tissue ratios, as shown in table 5.2, being greatest for DOPEG and MOPEG and least for VMA. Of the total metabolites

TABLE 5.2 Comparison of the distribution of metabolites from untreated rabbit uteri, separated by different chromatographic techniques.

		Total ^3H	NA + DOMA	DOPEG	VMA	MOPEG	NMN	ε^3H
T.L.C. (n = 10)	Tissue	17.33 ± 2.7	9.32 ± 1.86	1.43 ± 0.24	1.73 ± 0.46	1.20 ± 0.15	1.16 ± 0.09	14.84 (86%)
	Medium	-	-	6.66 ± 1.86	0.61 ± 0.16	4.57 ± 0.56	2.74 ± 0.38	-
	Total	-	-	8.09	2.34	5.77	3.92	-
	Medium / Total	-	-	4.66	0.35	3.81	2.36	-

		Total ^3H	NA	DOMA	DOPEG	VMA + MOPEG	NMN	ε^3H
DOWES 50/ ALUMINA (n = 8)	Tissue	17.08 ± 1.08	7.23 ± 0.85	0.24 ± 0.02	0.73 ± 0.03	3.04 ± 0.34	2.37 ± 0.44	13.71 (80%)
	Medium	-	-	0.59 ± 0.09	4.65 ± 0.62	7.51 ± 0.96	8.83 ± 1.46	-
	Total	-	-	0.83	5.38	10.55	11.20	-
	Medium / Tissue	-	-	2.46	6.37	2.47	3.73	-

Footnote: Metabolites are expressed as means ± S.E.s in nmol (g dry wt)$^{-1}$ (30 min)$^{-1}$ for tissue and medium contents.

formed in 30 min, DOPEG was the major metabolite (40%) followed by MOPEG (29%), NMN (19%) and VMA (12%). Deaminated metabolites represented 81% and o-methylated metabolites represented 60% of the total metabolites formed. 40% of the total metabolites were produced by the combined action of COMT and MAO (indicated by the combined formations of VMA and MOPEG).

(ii) *^{3}HdlNA*

The data in table 5.3 compare the distribution of metabolites produced by untreated rabbit uteri when segments from the same uteri were incubated with ^{3}HlNA and ^{3}HdlNA. Samples were analysed by TLC for all metabolites except DOMA which was measured by column chromatography. It may be noted that the results for tissues incubated with ^{3}HlNA agree well with those presented in table 5.2, except for tissue contents of VMA. This discrepancy may be due to the small sample number of 4 in table 5.3.

A comparison of the results obtained using different substrates showed that the total ^{3}H accumulated by the tissue was greater with ^{3}HlNA as substrate than with ^{3}HdlNA ($p < 0.05$, 2 tailed, paired t test). Although there was no significant difference in NA contents of the tissue, uteri incubated with ^{3}HdlNA had significantly lower tissue contents of DOPEG and DOMA than those incubated with ^{3}HlNA. The greatest difference in metabolite formation was seen with DOPEG which was produced in three times the quantity when ^{3}HlNA was the substrate than when ^{3}HdlNA was the substrate ($p < 0.05$, tissue, $p < 0.001$ medium). There was a tendency for the formation of MOPEG and DOMA to be greater in segments of uterus incubated with ^{3}HlNA although this difference did not attain significance at the 5% level ($0.05 < p < 0.10$).

TABLE 5.3 Comparison of the metabolism of different stereo-isomers of NA by untreated rabbit uteri.

		^{3}H dl NA (1.2 µmol l^{-1})	^{3}H l NA (1.2 µmol l^{-1})
Total ^{3}H accumulated (n = 6)		14.6 ± 1.5	16.91 ± 0.97
NA	Tiss (n = 4)	10.84 ± 2.49	9.47 ± 0.94
DOPEG	Tiss (n = 4)	0.22 ± 0.09	0.45 ± 0.10
	Med (n = 8)	1.61 ± 0.17	5.04 ± 0.56
VMA	Tiss (n = 4)	0.11 ± 0.10	0.16 ± 0.16
	Med (n = 8)	0.41 ± 0.19	0.49 ± 0.12
NMN	Tiss (n = 4)	1.47 ± 0.76	1.18 ± 0.51
	Med (n = 8)	2.37 ± 0.75	3.20 ± 1.36
MOPEG	Tiss (n = 4)	0.48 ± 0.26	0.80 ± 0.46
	Med (n = 8)	2.16 ± 0.28	4.70 ± 1.14
DOMA	Tiss (n = 4)	0.30 ± 0.10	0.61 ± 0.01
	Med (n = 4)	0.37 ± 0.06	1.71 ± 0.26
ε^3H		13.42 (92%)	12.68 (75%)

Footnote: metabolite were expressed as means ± S.E.S. in nmol. (g dry wt)$^{-1}$ (30 min)$^{-1}$ for tissue and medium contents.

These results suggested that a greater proportion of ^{3}H l NA was metabolized by the MAO pathway. Moreover more ^{3}H l NA was converted to metabolites since the total amounts of metabolites formed from ^{3}H l NA (18.5 nmol g^{-1} (30 min)$^{-1}$) greatly exceeded those formed from ^{3}H dl NA (9.6 nmol g^{-1} (30 min)$^{-1}$). The greater formation of DOPEG by ^{3}H l NA could be interpreted as evidence that DOPEG is neuronal in origin since l NA is accumulated to a greater degree than dl NA by nerve cells. Further evidence is presented below in experiments on the effect of cocaine on uterine metabolites.

(b) Ovariectomized Rabbits

In a group of ovariectomized rabbits (table 5.4) the distribution of uterine metabolites of ^{3}H l NA differed from that in uteri from untreated rabbits in that the proportion of DOPEG to the total (32%) was less and the proportion of NMN (38%) greater. The total ^{3}H accumulated by the tissue was also slightly but significantly lower in uteri from ovariectomized rabbits.

3. EFFECT OF ENZYME INHIBITORS

The effect of enzyme inhibitors was studied in ovariectomized animals. As shown in table 5.5, the COMT inhibitor, UO521 (55 μmol l^{-1}) caused an 87% reduction in NMN, 98% reduction in MOPEG and a 62% reduction in VMA formation. DOPEG formation increased markedly by 136%. The MAO inhibitor nialamide (350 μmol l^{-1}) reduced DOPEG formation by 88%, MOPEG by 81% and VMA by 55% while NMN was unaffected. For comparison data on the effects of the same inhibitors when examined by the column method is shown in table 5.6. There was a relatively small reduction in the NMN (66%) and VMA + MOPEG (51%) formation caused by UO521 and nialamide also had a relatively small effect on VMA + MOPEG

TABLE 5.4 Comparison of distribution of metabolites of ^{3}H l NA in uterine segments from ovariectomized rabbits and untreated rabbits.

TREATMENT		TOTAL ^{3}H	NA + DOMA*	DOPEG	VMA	NMN	MOPEG	$\varepsilon^{3}H$
UNTREATED (n = 10)	Tissue	17.33 ± 2.70	9.32 ± 1.86	1.43 ± 0.24	1.73 ± 0.46	1.16 ± 0.09	1.20 ± 0.15	14.84 (86%)
	Medium	-	-	6.66 ± 1.86	0.61 ± 0.16	2.74 ± 0.38	4.57 ± 0.56	-
	Total	-	-	8.09	2.34	3.92	5.77	-
	% Total Metabs.	-	-	40	12	19	29	-
OVARIEC-TOMIZED (n = 5)	Tissue	12.71 ± 1.24	7.55 ± 0.83	0.54 ± 0.07	0.61 ± 0.15	1.47 ± 0.36	0.59 ± 0.06	10.76 (85%)
	Medium	-	-	3.4 ± 0.50	0.46 ± 0.31	3.23 ± 1.03	1.95 ± 0.64	-
	Total	-	-	3.94 ± 0.49	1.07 ± 0.39	4.70 ± 1.10	2.54 ± 0.55	-
	% Total Metabs.	-	-	32	9	38	21	-

Footnote: Separation by TLC except *DOMA 0.24 nmol g^{-1} $(30\ min)^{-1}$ in tissue : total DOMA = 0.83 nmol g^{-1} $(30\ min)^{-1}$.

TABLE 5.5 Effect of enzyme inhibitors and cocaine on the metabolite distribution in uteri from ovariectomized animals.

TREATMENT		TOTAL ^3H	NA + DOMA	DOPEG	VMA	NMN	MOPEG	ε^3H
NIL	Medium	-	-	3.4 ± 0.5	0.46 ± 0.31	3.23 ± 1.03	1.95 ± 0.64	-
	Tissue	12.71 ± 1.24	7.55 ± 0.83	0.54 ± 0.07	0.61 ± 0.15	1.47 ± 0.36	0.59 ± 0.06	10.76 (85%)
	Total	-	-	3.94 ± 0.49	1.07 ± 0.39	4.70 ± 1.10	2.54 ± 0.55	-
U0521	Medium	-	-	6.59 ± 1.88	0.32 ± 0.17	0.61 ± 0.71	0.03 ± 0.002	-
	Tissue	17.2 ± 2.3	11.55 ± 1.40	2.71 ± 0.70	0.09 ± 0.04	0.01 ± 0.007	0.013± 0.004	14.37 (84%)
	Total	-	-	9.30 ± 1.73	0.41 ± 0.18	0.62 ± 0.60	0.04 ± 0.002	-
NIAL	Medium	-	-	0.23 ± 0.23	0.35 ± 0.24	3.62 ± 0.75	0.32 ± 0.17	-
	Tissue	16.87 ± 1.28	11.33 ± 1.27	0.25 ± 0.13	0.14 ± 0.04	2.21 ± 0.49	0.16 ± 0.075	14.09 (84%)
	Total	-	-	0.48 ± 0.25	0.48 ± 0.28	5.82 ± 0.80	0.49 ± 0.09	-
COCAINE	Medium	-	-	0.69 ± 0.33	0.43 ± 0.21	2.72 ± 0.71	1.43 ± 0.27	-
	Tissue	7.38 ± 0.93	3.81 ± 0.26	0.29 ± 0.23	0.32 ± 0.03	1.62 ± 0.62	0.51 ± 0.18	6.55 (89%)
	Total	-	-	0.80 ± 0.29	0.75 ± 0.18	4.34 ± 1.13	1.94 ± 0.39	-

Footnotes: 1) Separation of metabolites was by TLC.
2) metabolites expressed as nmol $(g\ dry\ wt)^{-1}\ (30\ min)^{-1}$.
3) n = 5

TABLE 5.6 Distribution of metabolites in uterine segments from untreated rabbits: Separation by column Chromatography.

Treatment		Total ^3H	NA	DOPEG*	DOMA*	DOMA + DOPEG	NMN	DOPEG + VMA	ε^3H
NIL (n = 9)	Tiss	17.08 ± 1.08	7.23 ± 0.85	0.73 ± 0.03	0.24 ± 0.02	1.07 ± 0.09	2.37 ± 0.44	3.04 ± 0.34	13.71 (80%)
	Med	-	-	4.65 ± 0.62	0.59 ± 0.08	6.06 ± 1.06	8.83 ± 1.46	7.51 ± 0.96	
	Total	-	-	5.38	0.83	7.13	11.2	10.55	
NIALAMIDE (n = 5)	Tiss	22.81 ± 2.32	12.4 ± 1.8	N.D.	N.D.	0.20 ± 0.05	4.60 ± 0.90	1.1 ± 0.2	18.3 (80%)
	Med	-	-			0	10.7 ± 3.7	3.59 ± 1.55	
	Total	-	-			0.2	15.3	4.69	
U0521 (n = 5)	Tiss	23.24 ± 1.40	9.4 ± 1.1	N.D.	N.D.	4.10 ± 0.60	0.7 ± 0.3	2.5 ± 0.4	16.7 (72%)
	Med	-	-			9.11 ± 2.66	3.13 ± 1.33	2.67 ± 1.46	
	Total	-	-			13.21	3.83	5.17	

Footnote: 1) Metabolites in nmol. (g dry wt)$^{-1}$ (30 min)$^{-1}$

2) * n = 5 for samples separated into DOPEG & DOMA.

formation (56% reduction) when examined by the column technique. The relatively poor effects of the enzyme inhibitors on the formation of these metabolites when the latter were assayed by the column technique was an important factor in rejecting this technique in favour of the TLC method. However, it should be noted that the column method appeared to provide adequate isolation of the DOMA + DOPEG fraction since their formation was decreased by 97% by nialamide. Moreover the values of DOPEG obtained by TLC and column chromatography were similar.

4. EFFECT OF COCAINE

The effect of cocaine on uterine metabolites was examined in ovariectomized rabbits. The data in table 5.5 indicated that the only metabolite significantly affected by cocaine was DOPEG, whose formation was reduced by 80%. The tissue content of NA + DOMA was reduced by 50%. This result was interpreted as evidence that DOPEG was mainly of neuronal origin in uteri from ovariectomized rabbits. The effect on ^{3}HNA + DOMA content of the tissue was smaller than would be expected if these were entirely neuronal in origin and indicated that approximately 40-50% of this fraction in the tissue was non-neuronal.

5. THE OVARY AND OVIDUCT

The distribution of metabolites of ^{3}HlNA produced by the uterus, ovary and oviduct in untreated rabbits is compared in table 5.7. All metabolites were separated by TLC. Of the total metabolites formed, ovaries produced relatively more NMN (51%) and relatively less DOPEG (28%) and MOPEG (10%) than uteri. In contrast, the oviduct generated

TABLE 5.7 Comparison of metabolites produced by rabbit uterus, ovaries and oviducts.

		METABOLITES						
		TOTAL ^3H	NA + DOMA	DOPEG	VMA	NMN	MOPEG	ε^3H
UTERUS	Tiss	17.33 ± 2.7	9.32 ± 1.86	1.43 ± 0.24	1.73 ± 0.46	1.16 ± 0.09	1.20 ± 0.15	14.84 (86%)
	Med	-	-	6.66 ± 1.86	0.61 ± 0.16	2.74 ± 0.38	4.57 ± 0.56	
	Total	-	-	8.09	2.34	3.92	5.77	
	% Total Metabs.	-	-	40	11.6	19	29	
OVARY	Tiss	9.37 ± 3.34	4.40 ± 1.71	0.38 ± 0.08	0.82 ± 0.22	1.49 ± 0.46	0.38 ± 0.10	7.47 (80%)
	Med	-	-	2.48 ± 0.79	0.24 ± 0.19	3.76 ± 1.30	0.68 ± 0.19	
	Total	-	-	2.86	1.06	5.25	1.06	
	% Total Metabs.	-	-	28	10.4	51	10.4	
OVIDUCT	Tiss	8.86 ± 1.45	6.75 ± 1.07	0.42 ± 0.05	0.56 ± 0.24	0.10 ± 0.01	0.26 ± 0.03	8.09 (91%)
	Med	-	-	5.96 ± 1.05	0.25 ± 0.04	0.44 ± 0.02	0.89 ± 0.04	
	Total	-	-	6.38	0.81	0.54	1.15	
	% Total Metabs.	-	-	72	9	6	13	

Footnote 1. All metabolites were separated by TLC : n = 5 for ovaries and oviducts
n = 10 for uterus.

2. Metabolites in nmol. (g dry wt)$^{-1}$, (30 min)$^{-1}$.

mainly DOPEG (72%). The total ^{3}H and NA + DOMA contents of the tissue in ovaries and oviducts were less than those of the uterus when expressed on a dry weight basis. In the ovary the tissue contents of ^{3}H and NA + DOMA were very variable. On a wet weight basis the ^{3}H content of ovaries (1.37 nmol g^{-1} (30 min)$^{-1}$) was slightly greater than that of the medium (1.2 nmol ml^{-1}) but unchanged NA was less than in the medium being 0.64 ± 0.25 nmol (g wet wt)$^{-1}$ (30 min)$^{-1}$. Since the oviduct did not have as high a water content as the uterus (wet/dry wt = 4.26 ± 0.20; n = 8) when expressed per wet wt of tissue, the contents of ^{3}H (2.08 ± 0.34 nmol g^{-1} (30 min)$^{-1}$) and NA + DOMA (1.58 ± 0.25 nmol g^{-1} (30 min)$^{-1}$) in oviducts were comparable with those in the uterus.

Fig. 5.1 summarises the effect of cocaine on the ovarian metabolites. Cocaine significantly reduced the formation of DOPEG by 70% ($p < 0.05$). It had no effect on the total ^{3}H content of ovaries nor on the tissue content of NA + DOMA suggesting a predominantly extraneuronal site for the relatively small amount of NA taken up by this tissue (4.40 nmol (g dry wt)$^{-1}$ (30 min)$^{-1}$, table 5.7). It should be noted that the S.E. for the NA + DOMA fraction in ovaries was large. This probably reflects the small sample size of 4 for tissue contents. Cocaine had no effect on the formation of the other metabolite fractions.

In rabbit oviducts, cocaine decreased DOPEG formation by 92% ($p < 0.02$, fig. 5.2). The tissue content of ^{3}H was significantly reduced by cocaine as was the NA + DOMA content. The decrease in NA + DOMA was 80% ($p < 0.02$), suggesting a predominantly neuronal site of accumulation of NA in this tissue. The formations of the other metabolites were not reduced by cocaine, NMN being significantly increased (by 150%, $p < 0.005$).

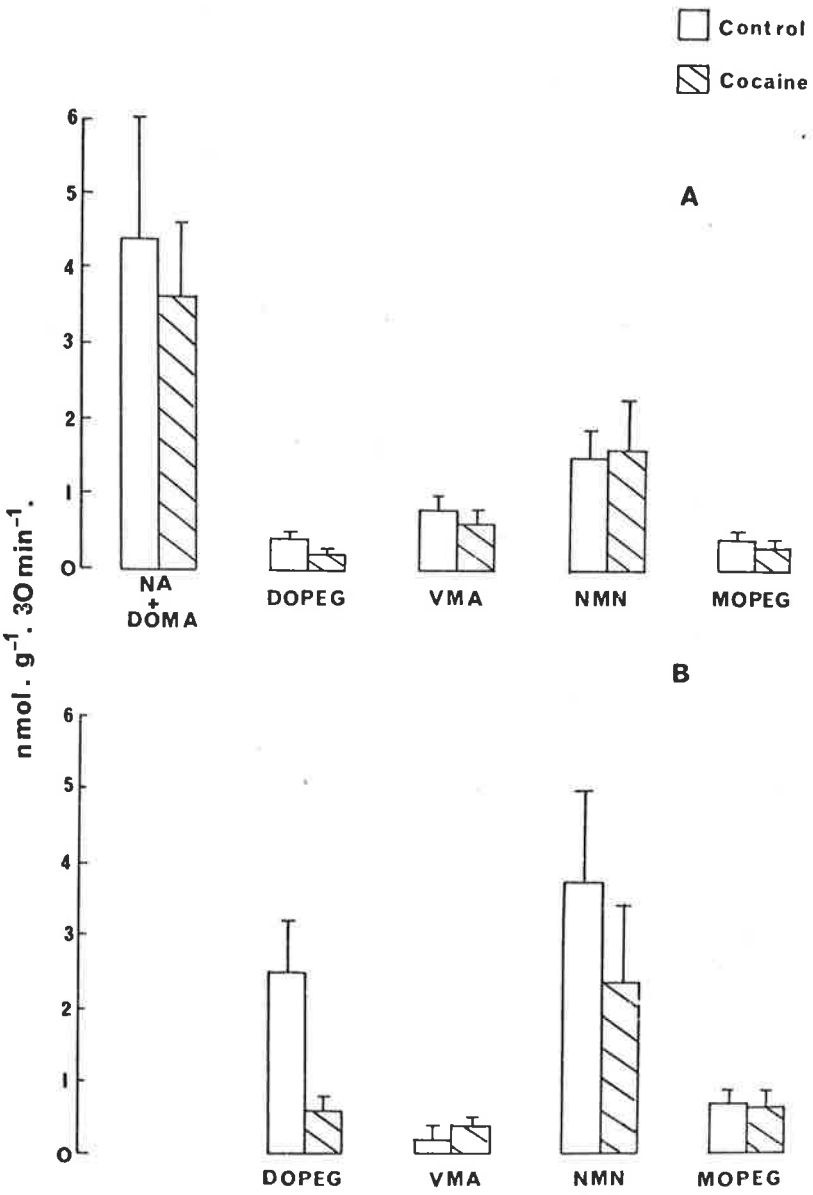

Fig. 5.1 The effect of cocaine (29 μmol l^{-1}) on the metabolites of ^{3}H l NA present in tissues (A) and incubating media (B) after incubation of ovaries from untreated rabbits. Values represent means + SEs of 4 comparisons. Metabolites were separated by TLC. Cocaine significantly decreased DOPEG formation ($p < 0.05$, paired t test).

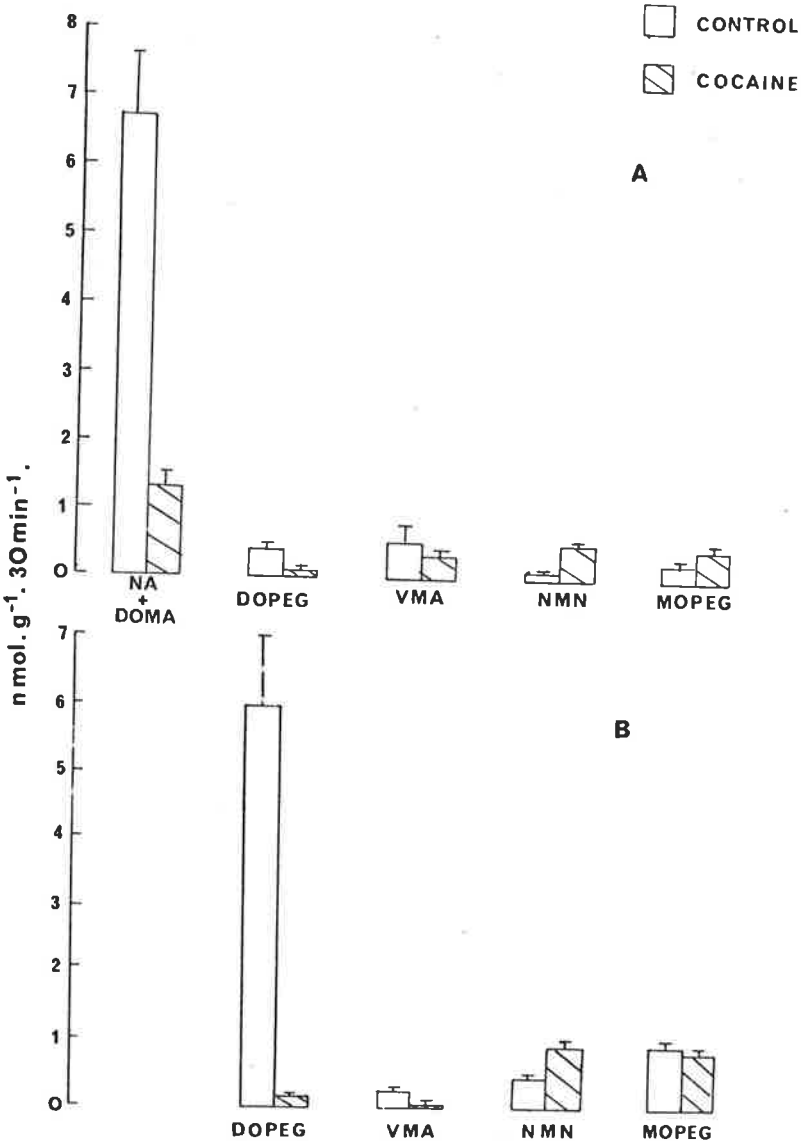

Fig. 5.2 The effect of cocaine on the distribution of metabolites of ^{3}H] NA present in tissues (A) and incubating media (B) after incubation of oviducts from untreated rabbits. Values represent means ± SEs of 4 comparisons. Cocaine significantly decreased DOPEG formation ($p < 0.02$) and increased NMN formation ($p < 0.005$, paired t test).

DISCUSSION

1. UPTAKE OF ^{3}H] NA

The results indicate that the rabbit uterus can accumulate NA against a concentration gradient but that the accumulation is small (1.5 fold) in magnitude. The tissue to medium ratio based on total ^{3}H accumulation is greater (1.96 fold) but this reflects the fact that 40% of the ^{3}H is in the form of metabolites of NA.

Compared to other tissues, the accumulation of ^{3}H] NA and ^{3}H in the uterus appears comparable with that of the oviduct but considerably greater than that of the ovary. However it should be noted that NA was not measured separately from DOMA in the oviduct and ovary and it is possible that the proportion of DOMA to NA may have been greater in the oviduct and ovary. However, if this were so, the accumulation of NA by the ovary would be even less compared with the uterus.

The tissue to medium ratio of ^{3}H] NA in the rabbit uterus is considerably lower than in the rabbit ear artery (3.0, Head 1976) and the aorta (1.5, Levin, 1974). The poorer accumulation of NA by the uterus compared with the ear artery reflects its poorer sympathetic innervation indicated by its lower endogenous NA content (2.88 nmol g^{-1} cf. 19.0 nmol g^{-1} respectively). The aorta has a lower endogenous NA content (1.5 nmol g^{-1}) than the uterus but its greater accumulation of ^{3}H] NA may be due to the fact that the sympathetic nerve terminals are concentrated on the outside surface of the aorta in the adventitia thereby affording NA greater access to the sympathetic nerves in the aorta than in the uterus where the nerve terminals are distributed throughout the tissue.

Cocaine decreased the accumulation of ^{3}Hl NA in the uterus by 50%. There is considerable evidence that cocaine inhibits neuronal uptake of NA in other tissues (Iversen, 1967). Moreover the concentration of cocaine used here has been shown by Trendelenburg *et al.* (1972) to inhibit neuronal uptake of NA in cat nictitating membrane and by Head *et al.* (1975) to decrease the uptake of NA in the rabbit ear artery by 75% and to produce effects on the metabolites of NA comparable to the effects of chronic denervation. Assuming that cocaine inhibits neuronal uptake of NA in the uterus as it does in other tissue, the cocaine - sensitive accumulation may be taken as a measure of the extent of uptake of NA by the nerves, and hence will also be related to the density of innervation. This arguement is consistent with the much greater uptake of NA by the ear artery under comparable conditions (8.7 nmol g^{-1}, Head *et al.* 1975), 75% of which was sensitive to cocaine. It is also consistent with the present evidence that the oviduct accumulated more cocaine-sensitive NA (1.26 nmol g^{-1} wet wt.) than the uterus (0.81 nmol g^{-1} wet wt.) and also possessed a higher endogenous NA content than the uterus (3.41 nmol g^{-1} cf 2.88 nmol g^{-1} respectively). Moreover, the ovary possessed the lowest endogenous NA content (1.18 nmol g^{-1}) of all 5 tissues and took up the smallest amount of NA. In the ovary the NA taken up is insensitive to cocaine as though the NA is located extraneuronally.

In summary, the rabbit uterus has a relatively low endogenous NA content and barely accumulates ^{3}Hl NA above its concentration gradient. Increased accumulation of ^{3}Hl NA appears to be associated with increased sympathetic innervation as indicated by a higher endogenous NA content.

2. METABOLITES - VALIDITY OF TLC FRACTIONATION

As shown in the results section, the MAO and COMT inhibitors produced greater inhibition of the formation of the various metabolite fractions when the latter were assayed by the TLC method. The poorer resolution by the column technique for all of the fractions except DOPEG and DOMA probably reflects the greater cross-over of ^{3}H from the NA fraction for o-methylated fractions separated by this technique (see Chapter 3). This error would be greatest in the case of the incubating media where the concentration of NA relative to metabolites was much higher than in the tissue extracts. As shown in table 5.2, this was the case, discrepancies between the results obtained by the two techniques being greatest for assays of the media.

3. METABOLITE DISTRIBUTION

In the untreated rabbit uterus, 81% of the total metabolites were in the form of deaminated metabolites and 60% in the form of o-methylated metabolites, indicating that both the MAO & COMT pathways played major roles in the metabolism of NA. A similar conclusion applies to the distribution in uteri from the ovariectomized animals since 62% was deaminated and 68% was o-methylated. Of the deaminated metabolites DOPEG and MOPEG predominated while the o-methylated metabolites were mainly MOPEG and NMN. In the untreated uterus the distribution was altered when dl NA was the substrate, the o-methylated fractions being increased to 74% of the total ^{3}H metabolites and the deaminated fractions decreased to 54% of the total. This change in metabolite distribution was due to the smaller amount of DOPEG produced by uteri incubated with dl NA. The stereo specificity of metabolite formation is not surprising when the effect of cocaine on the uterine metabolites from ovariectomized animals is considered. The major effect of cocaine was to decrease DOPEG

formation by 80% thereby altering the proportion of deaminated metabolites to 45% of the total and o-methylated metabolites to 90% of the total ^{3}H metabolitesi.e. cocaine alters the metabolite distribution in the same direction as incubating the tissue with dl NA. Hence the difference in metabolism of l and dl NA can be interpreted as evidence that, in the uterus, the l form is preferentially metabolised by the sympathetic nerves. In this respect the rabbit uterus resembles the rabbit aorta where Levin (1974) showed preferential metabolism of the l form to DOPEG. DOPEG was also the main metabolite produced by the isolated adventitia of the aorta which is the region of this artery containing the sympathetic nerves. Eckert *et al.* (1976) have subsequently shown that there was preferential binding of ^{3}Hl NA over ^{3}Hd NA by storage vesicles in UO521 and pargylene inhibited aortic strips which accounted for the greater initial rate of accumulation of lNA compared with dNA. No difference in d and lNA accumulation was evident in nerve-free strips. They also observed a stereoselective metabolite pattern for late effluxing metabolites from nerves in that more DOPEG was produced by ^{3}Hl NA and more DOMA and NMN by ^{3}Hd NA.

4. SOURCE OF METABOLITES

As indicated in the preceding discussion, the decrease in DOPEG formation by cocaine can be viewed as evidence that the major proportion of this metabolite (80%) was neuronal in origin in uteri from ovariectomized rabbits. Equally the lack of an effect on the remaining uterine metabolites can be viewed as evidence that these were extra-neuronal in origin. This result is comparable to the data of Levin (1974) for the rabbit aorta mentioned above, that of Head (1976) for rabbit ear artery and of Graefe *et al.* (1973) for rat vas deferens. In each

of these tissues, DOPEG is the main metabolite of INA produced in the sympathetic nerves. Similarly in these and other tissues the o-methylated metabolites appear to be largely extraneuronal, the only important exception being the cat nictitating membrane in which NMN and MOPEG are partly neuronal in origin (Graefe and Trendelenburg 1974, Jarrot and Langer 1971 and Langer *et al.* 1972).

5. RELATIVE CONTRIBUTION OF MAO AND COMT PATHWAYS

It was shown in chapter 4 that the activity of COMT in uterine homogenates (1.35 ± 0.06 nmol (mg protein)$^{-1}$ hr^{-1}) was much less than MAO activity (513 ± 100 mmol (mg protein)$^{-1}$ hr^{-1}). The fact that quantitatively COMT is as important as MAO in the metabolism of NA in the intact-cell uterine preparation can be explained in a variety of ways e.g.

a) The activity of the enzyme itself in the intact cell mignt differ from that in homogenates in that it is conceivable that its conformation may be altered by isolating it from other cell components. In support of this, it has been shown that COMT in the intact rat heart has a lower Km than that reported for most purified preparations of COMT from a number of tissues (Trendelenburg 1976). Furthermore, there is some evidence that COMT may exist in a particulate form in rat erythrocytes which differs from the soluble form in that it has a different pH optimum, heat stability and immunochemical reactivity (Bohuon and Assicot, 1973).

b) There is a great deal of evidence to show that NA is transported into the target organ cells (nerve or smooth muscle) by specific transport systems (Iversen, 1967) and these may lead to high localized concentrations of substrate available to the enzymes. The extent of

substrate availability to MAO and COMT may differ as a result of differences in the activity of these transport systems.

c) The specificity of the enzymes for the substrate, NA, may depend on the relative proportions of the different forms of the enzymes within the tissue. In the present case, MAO activity was estimated using tyramine as a substrate. Since tyramine is metabolized by both A and B type isoenzymes of MAO and NA has a high affinity only for the A type (Youdim 1972) it is difficult to assess the relative contribution of MAO and COMT to the metabolism of NA based on this estimate of MAO activity.

Apart from the more obvious evidence of a series relationship between COMT and MAO activities provided by the fact that some metabolites require both enzymes, another relationship is apparent from finding that inhibition of COMT led to a 2.5 fold increase in DOPEG formation. The reverse effect was much less apparent in that in the MAO inhibited uterus, the formation of NMN was greater than in controls but not significantly so. These results may be explained in several ways. Either the increase in DOPEG reflects its lack of further metabolism to MOPEG by COMT or alternatively it reflects an increased availability of substrate to MAO in the absence of COMT activity (see fig. 5.3). Both explanations may apply since the decrease in MOPEG (2.5 nmol g^{-1}) when added to the DOPEG content of the untreated uterus (3.94 nmol g^{-1}) is still less (6.44 nmol g^{-1}) than the increased production of DOPEG (9.30 nmol g^{-1}) in the COMT inhibited uterus. The failure of inhibition of MAO to significantly increase formation of o-methylated metabolites, particularly NMN suggests that either NMN is normally not further metabolized to MOPEG or VMA in this tissue, or that inhibition of MAO

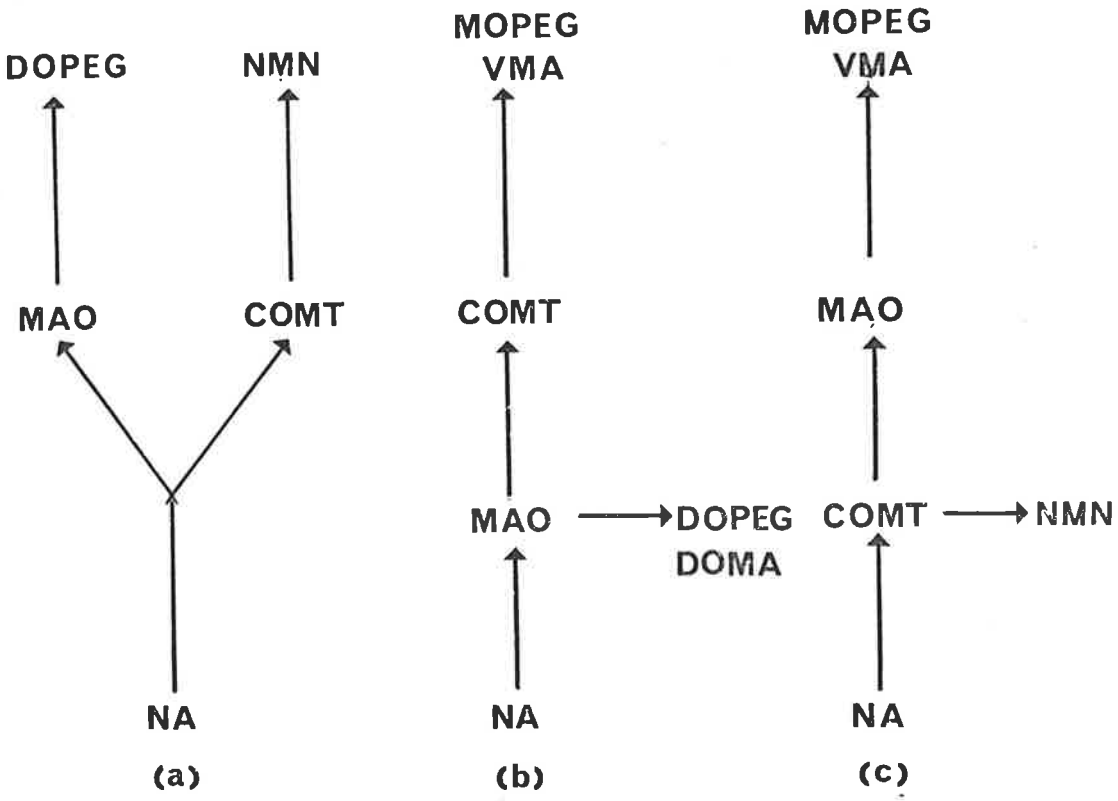

Fig. 5.3 Possible arrangements of MAO and COMT in the rabbit uterus.

(a) represents a parallel arrangement of enzymes;

(b) represents alternative schemes for the formation of o-methylated deaminated metabolites of NA by a series arrangement of enzymes.

does not increase the amount of substrate available to COMT. The latter situation might be explained if the formation of DOPEG was highly localized in small areas of the tissue, such as the sympathetic nerve terminals. Although decreasing the amount of substrate available to COMT in the immediately adjacent cells, the ratio of volume of terminals to smooth muscle mass is probably so small that cells further away from the terminals would be little affected. This concept is supported by the finding that cocaine, while inhibiting DOPEG formation, also failed to increase NMN formation.

6. COMPARISON WITH OTHER RABBIT TISSUES

(a) Reproductive Tissues

(i) Ovary

The rabbit ovary accumulated and metabolized less ^{3}H NA than the uterus (10.2 nmol g^{-1} dry wt cf. 20.9 nmol g^{-1} dry wt respectively). It appears that o-methylation is quantitatively more important than in the uterus since NMN was the major metabolite in the ovary representing 51% of the total metabolites. VMA was proportionately the same as in the uterus while both MOPEG and DOPEG were produced in lower proportions. As in the uterus, only DOPEG formation appeared neuronal, since cocaine markedly decreased its formation but had no effect on the other metabolites. Hence extraneuronal metabolism predominates over neuronal metabolism to an even greater degree than in the uterus. The NA taken up by the ovary appears to be largely extraneuronal since cocaine was without effect on the NA + DOMA fraction in tissue extracts. The apparently

small extent of neuronal accumulation and metabolism of ^{3}HlNA in the ovary is not surprising since it has a much lower endogenous NA content than the uterus (1.76 nmol g^{-1} cf. 2.88 nmol g^{-1} respectively) implying a smaller degree of innervation. The different accumulation may also reflect differences in access of NA to the sympathetic nerves in the two tissues. In both tissues the distribution of nerve terminals is not uniform. In both tissues, the nerves are associated with blood vessels, but in addition, in the ovary they are associated with follicles and in the uterus with smooth muscle cells of the myometrium (Marshall, 1970).

(ii) Oviduct

In complete contrast to the ovary, the oviduct forms mainly DOPEG which represents 71% of the total metabolites. Its formation was virtually eliminated by cocaine, being decreased by more than 90%. Furthermore, the NA + DOMA content of the tissue was decreased by 80% by cocaine. Thus, although the total ^{3}H accumulated per g wet weight of oviduct tissue was similar to the uterus, most of the ^{3}H was accumulated neuronally. It may also be concluded that neuronal oxidative deamination is the major pathway for metabolism in the oviduct. This accords well with the denser innervation displayed by this tissue, its NA content being 4.88 nmol g^{-1}. The innervation of the rabbit oviduct is also not uniform, there being a greater concentration of nerve terminals in the isthmus and utero-tubal junction (Brundin, 1966). Whether the accumulation and metabolism of NA is situated in one or both of these two regions is not known. In view of the high proportion of metabolism mediated neuronally, it is of great interest that elimination of this pathway by cocaine, leads to a diversion of metabolism of NA to

TABLE 5.8 Comparison of the Metabolite Distribution in 5 Rabbit Tissues.

Tissue	Endogenous NA Content (nmol g^{-1})	% of Total Metabolites				
		DOPEG	NMN	**DOMA	MOPEG	VMA
OVARY	1.76	28	51	N.D.	10	10
AORTA (WHOLE)	*1.50	30	50	5	12	3
UTERUS	2.88	39	19	3	28	11
OVIDUCT	4.88	71	6	N.D.	13	10
EAR ARTERY	19.0	68	10	9	8	5

Footnotes:

1. Data for ear artery from Head (1976); data for aorta from Levin (1974).
2. * Other endogenous NA contents have been reported for rabbit aorta e.g. 4.6 nmol g^{-1} by Bevan et al. (1972).
3. ** DOMA was estimated by column chromatography.

the extraneuronal pathway, as indicated by the significant increase in NMN. In the uterus and ovary where extraneuronal metabolism predominates, cocaine treatment did not lead to an increase in NMN.

In conclusion, in the rabbit uterus and ovary extraneuronal metabolism of NA predominates while in the oviduct neuronal metabolism predominates. In view of the non uniform distribution of nerves in these three tissues a study of the metabolism of NA by different areas of the tissues seems warranted.

(b) Arteries

Data on the three reproductive tissues are compared with the rabbit ear artery and aorta in table 5.8. Four of the tissues were incubated and analysed in an identical fashion, the exception being the data for the aorta taken from Levin (1974); the aorta was incubated with 3 μmol l^{-1} NA and its metabolites were separated by paper chromatography. There appears to be a relationship between DOPEG formation, and the sympathetic nerve density, as judged by the endogenous NA contents. Thus the ear artery has the densest innervation and the highest proportion of DOPEG to the total metabolites while the ovary has the least dense innervation and the lowest proportion of DOPEG. However it is of interest to note that of all the tissues in table 5.8, the pattern of distribution of metabolites in the ovary is similar to that in the aorta, while that of the oviduct is similar to that of the ear artery. An association of DOPEG formation with neuronal content has been demonstrated in the rabbit aorta by Levin (1974) as mentioned previously. The adventitia, which contains the nerve terminals, produced mainly DOPEG while the media, which possesses virtually no nerve terminals produced mainly NMN.

It should be noted that when compared to the other tissues in table 5.8 a distinguishing feature of the metabolism in the uterus is the relatively high proportion of MOPEG produced indicating that extra-neuronal glycol formation is more important in this tissue than in other rabbit tissues described to date.

In this chapter no account of variations in the weight of the uterus has been considered since all results were expressed per g weight of tissue. In the following chapter dealing with the effect of steroids the relevance of the change in uterine weight to its metabolite distribution will be considered.

CHAPTER 6

THE EFFECT OF OVARIAN STEROIDS ON THE METABOLISM OF NORADRENALINE BY RABBIT UTERINE SEGMENTS

The data in Chapter 4 indicated that the activities in uterine homogenates of both MAO and COMT increased after pretreatment with 17β-oestradiol whereas treatment with progesterone alone increased only MAO activity. When progesterone treatment followed oestrogen priming the activity of both enzymes was increased. The experiments in this chapter were designed to investigate whether these changes in enzyme activity were manifested as changes in the formation of the corresponding metabolites of NA in uterine segments in which cell structure and tissue integrity are preserved. In this way, it was hoped to obtain a more realistic indication of the effect of ovarian steroids on the metabolism of NA since in the uterine segments the influence of the cellular uptake processes on access of the substrate to the enzymes is retained. That the cellular uptake processes may be important in the action of both oestrogen and progesterone on the metabolism of NA by the intact uterus was suggested by the data of Iversen & Salt (1970), who showed that these two steroids, as well as various corticosteroids, inhibited extraneuronal uptake of NA in the rat heart.

It should be noted that uterine segments contain myometrium, endometrium and the intrinsic blood vessels of the uterus and no attempt has been made in this study to separate the individual components.

METHODS

Rabbits were ovariectomized and pretreated with 17β oestradiol and progesterone according to the same regime as that described in Chapter 4. Two series of experiments were performed. In the first series the following three treatment groups were compared -

1) ovariectomized controls

2) 17β oestradiol pretreated

3) 17β oestradiol + progesterone pretreated

Rabbit ear arteries and segments of uterus were prepared for incubation as described in Chapter 3. They were incubated with ^{3}HINA as described in Chapter 2 and then washed for 1 minute in amine-free Krebs solution before extraction of ^{3}H metabolites.

In the second series of experiments, the same three treatment groups, plus an additional group receiving only progesterone, were compared. The main purposes of the second series were to increase the number of comparisons between treatment groups and to investigate the effect of progesterone in the absence of oestrogen priming. In addition, in series 2, samples were separated by column chromatography as well as TLC, in order to estimate the individual NA and DOMA contents of tissue extracts. Moreover, since it was possible that there may have been sufficient loss of metabolites during the one minute wash period used in series 1 to alter their pattern of distribution within the tissue, tissues were washed for only 10 secs. in series 2. This, of course, prevented pooling of data for tissue extracts from series 1 and 2 but did not preclude pooling of data for the incubating media.

A further difference between series 1 and 2 was that the uteri from ovariectomized control animals in series 2 were much smaller than those in series 1, necessitating sections of uterus longer than 1 mm in order to obtain 40 mg of tissue. The discrepancy in uterine size between the control animals in the two series was thought to be due to animals in series 2 being approximately 3 weeks younger than those in series 1 at the time of ovariectomy. However, all animals were sexually mature at that time.

RESULTS

1. METABOLITES OF ^{3}H l NORADRENALINE

(a) Series 1

(i) Effect of Ovarian Steroids

The data in table 6.1 indicate that the major metabolites produced by uteri per g of tissue from ovariectomized control rabbits were NMN (4.7 nmol g^{-1} $30min^{-1}$) and DOPEG (3.9 nmol g^{-1} $30min^{-1}$).

Although total ^{3}H accumulated by uterine tissue was similar for the three groups, the uteri from animals treated with steroids tended to metabolize more ^{3}H lNA than those from the controls. Metabolites represented 18.1 nmol g^{-1} $(30min)^{-1}$ for the combined steroid group, 14.5 nmol g^{-1} $(30min)^{-1}$ for the 17β oestradiol treated group and 12.3 nmol g^{-1} $(30min)^{-1}$ for the ovariectomized controls.

The effect of 17β oestradiol pretreatment on uterine metabolites was a significant increase (78%) in the formation of DOPEG. This increase in DOPEG formation was apparent only in the incubating medium tissue levels being comparable to controls. No other metabolite fraction was affected by 17β oestradiol pretreatment. Tissue values for NA + DOMA were also comparable to controls.

When progesterone treatment was combined with 17β oestradiol treatment on the oestrogen primed uterus, the most striking effect was a significant increase (123%) in NMN formation above the levels in control uteri and in those pretreated only with 17β oestradiol. This increase occurred in both the tissue and the incubating medium. The production of MOPEG was significantly increased (75%) in uteri from rabbits treated with progesterone + 17β oestradiol when compared with those

TABLE 6.1. Effect of ovarian steroids on metabolites of ^{3}HINA in rabbit uterine segments. Series 1.

TREATMENT		TOTAL ^{3}H	NA + DOMA	DOPEG	VMA	NMN	MOPEG	Σ^{3}H TISSUE	Σ^{3}H METABS.
Ovariectomized controls	Tissue	12.7±1.2	7.6±0.8	0.5±0.1	0.6±0.1	1.5±0.4	0.6±0.1	10.8±0.8 (85%)	-
	Medium	-	-	3.4±0.5	0.5±0.3	3.2±1.0	2.0±0.6	-	-
	Total	-	-	3.9±0.5	1.1±0.4	4.7±1.2	2.5±0.6	-	12.3
17β oestradiol	Tissue	10.6±3.2	7.3±1.8	0.4±0.03	0.4±0.1	1.1±0.5	0.7±0.1	10.0±1.4 (95%)	-
	Medium	-	-	6.6±0.8	1.1±0.4	2.3±0.9	1.7±0.4	-	-
	Total	-	-	**7.0±0.7	1.5±0.4	3.5±1.2	2.4±0.5	-	14.5
Progesterone + 17β oestradiol	Tissue	13.6±0.7	**4.6±0.6	0.4±0.05	0.9±0.3	4.3±1.7	1.0±0.2	11.2±1.5 (82%)	-
	Medium	-	-	1.3±0.8	0.8±0.3	6.2±1.2	3.2±0.3	-	-
	Total	-	-	*1.7±0.7	1.7±0.5	*10.5±2.4	4.2±0.4	-	18.1

Footnotes : 1) metabolites expressed in nmol (g dry wt)$^{-1}$(30 min)$^{-1}$

2) n = 5

3) * $p < 0.05$
** $p < 0.02$ unpaired 2 tailed t test, comparisons made with ovariectomized controls.

4) the MOPEG formation in progesterone + 17β oestradiol group was not significantly different from controls ($0.05<p<0.10$) but was significantly different from 17β oestradiol group ($p < 0.05$).

TABLE 6.2. Effect of ovarian steroids on metabolites of ^{3}HNA in rabbit ear arteries. Series 1.

TREATMENT		TOTAL ^{3}H	NA + DOMA	DOPEG	VMA	NMN	MOPEG	Σ^{3}H IN TISSUE
Ovariectomized control	Tissue	5.5±1.5	4.4±0.2	0.2±0.1	0.07±0.04	0.07±0.03	0.03±0.01	4.7 (85%)
	Medium	-	-	1.9±0.6	0.3 ±0.2	0.3 ±0.1	0.2 ±0.1	-
	Total	-	-	2.1±0.7	0.4 ±0.2	0.4 ±0.2	0.2 ±0.1	-
17β oestradiol	Tissue	7.3±0.8	4.9±0.5	0.2±0.04	0.06±0.03	0.05±0.02	0.03±0.01	5.3 (73%)
	Medium	-	-	2.0±0.7	0.4 ±0.2	0.2 ±0.06	0.2 ±0.02	-
	Total	-	-	2.2±0.7	0.4 ±0.3	0.3 ±0.1	0.3 ±0.03	-
Progesterone + 17β oestradiol	Tissue	7.7±1.8	5.0±0.7	0.2±0.06	0.07±0.05	0.05±0.02	0.04±0.01	5.4 (70%)
	Medium	-	-	1.8±0.1	0.4 ±0.2	0.3 ±0.1	0.3 ±0.1	-
	Total	-	-	2.0±0.2	0.5 ±0.2	0.4 ±0.1	0.3 ±0.1	-

Footnotes: 1) values are in nmol (g wet wt)$^{-1}$(30 min)$^{-1}$

2) n = 5

receiving only oestrogen. However, when compared with uteri from control animals the difference was not significant. The progesterone treatment also significantly decreased DOPEG formation per g. uterine tissue when compared with those in the control group and when compared with those treated with oestrogen alone. VMA formation was not affected. The amount of NA + DOMA in uterine tissue from rabbits receiving progesterone was significantly reduced below the level in ovariectomized controls.

Table 6.2 contains corresponding data for ear arteries from the same rabbits. Neither of the steroid treatment regimes had any significant effect on any metabolite fraction or on the tissue levels of ^{3}H or NA + DOMA.

(ii) Effect of Enzyme Inhibitors and Cocaine

The effects of nialamide and UO521 on metabolite formation were investigated in the three treatment groups in order to test whether a decrease in the activity of one enzyme pathway could account for some of the increases observed in the alternate enzyme pathway.

The effect of cocaine was investigated 1) to determine the neuronal or extraneuronal origin of metabolites in the three treatment groups and 2) to test whether a decrease in the neuronal pathway for inactivating NA could influence the activity of extraneuronal inactivation.

The results of these three drug treatments are shown in figs. 6.1, 6.2 and 6.3. The effect of cocaine (fig. 6.3) indicated that DOPEG was largely formed intra neuronally except for the small amount formed in uteri from the 17β oestradiol + progesterone groups which was relatively insensitive to cocaine. Moreover o methylated metabolites appeared to be produced extraneuronally in all groups.

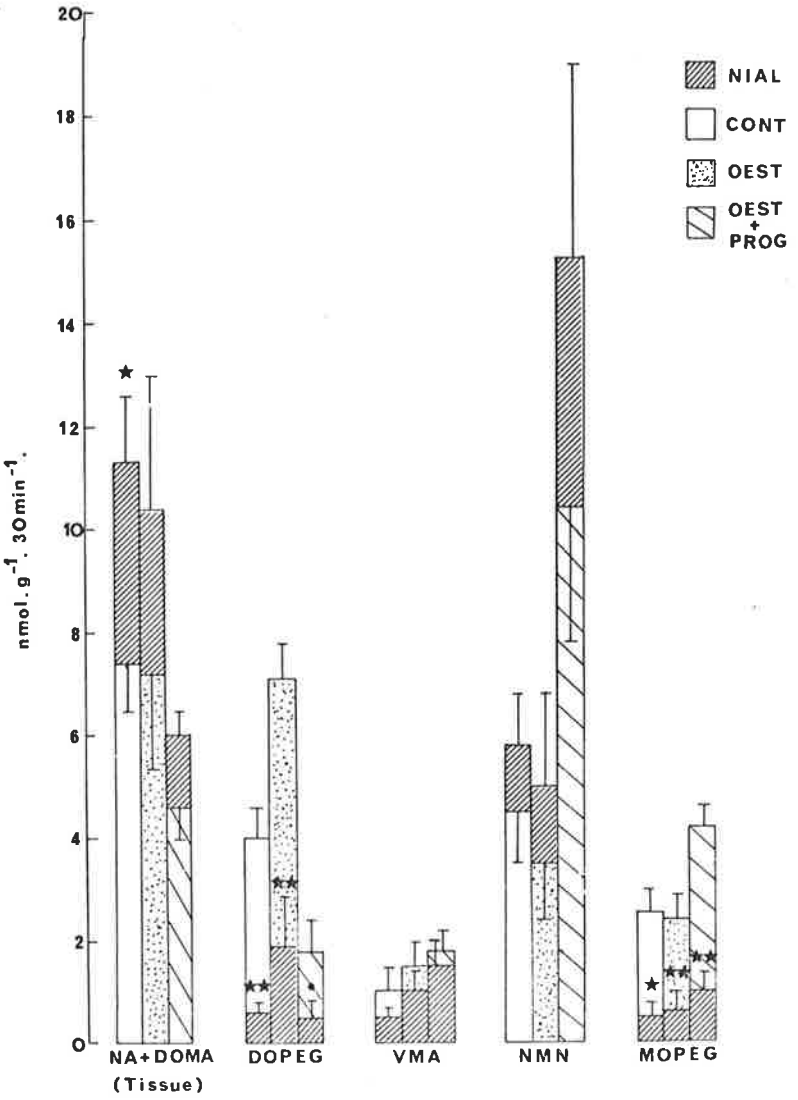

Fig. 6.1 The effect of nialamide (350 μmol l^{-1}) on formation of metabolites of ^{3}H l NA in rabbit uteri. Values are means ± SEs of 4 experiments. NA + DOMA was measured in tissues; other metabolites represent tissue + medium contents. * $p < 0.05$, ** $p < 0.005$, ● $0.05 < p < 0.10$ (2-tailed paired t test).

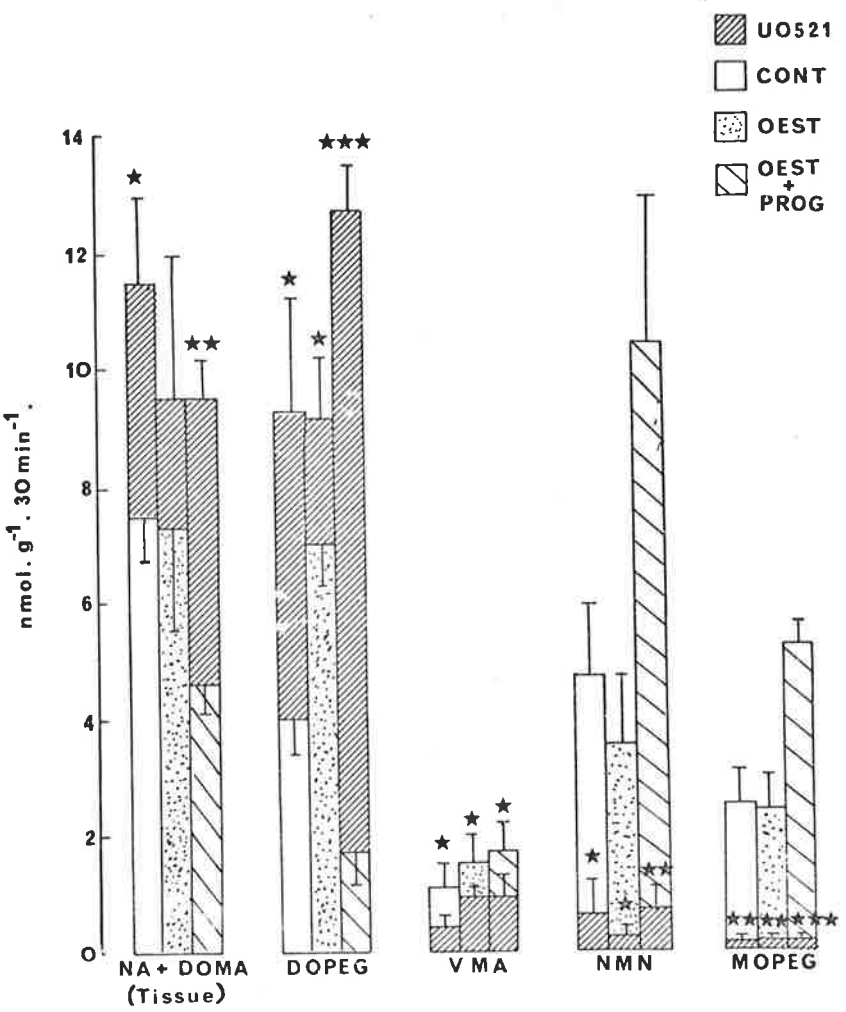

Fig. 6.2 The effect of UO521 (55 μmol l^{-1}) on the formation of metabolites of ^{3}H l NA in rabbit uteri. Values are means ± SEs of 4 experiments. NA + DOMA was measured in tissues; the other metabolite values represent tissue + medium contents. * $p < 0.05$, ** $p < 0.02$, *** $p < 0.005$ (2 tailed paired t test).

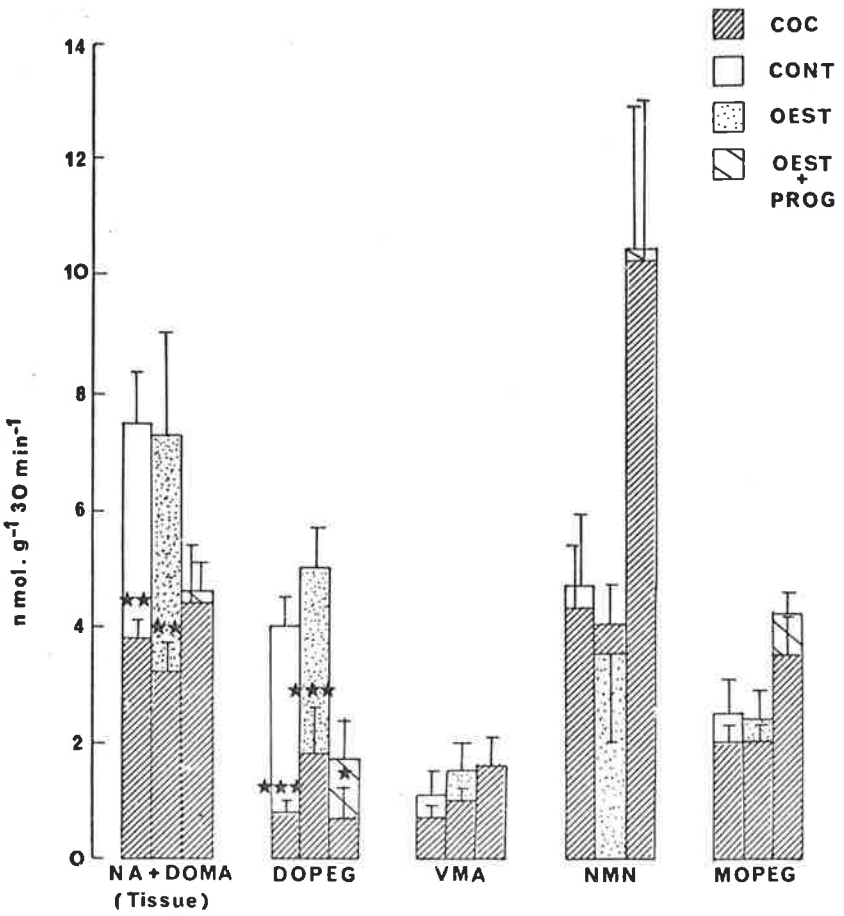

Fig. 6.3 Effect of cocaine (29 µmol l^{-1}) on the formation of metabolites of ^{3}H l NA in rabbit uteri. Values are means ± SEs of 4 experiments. NA + DOMA was measured in tissues; the other metabolite values represent tissue + medium contents. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$ (2 tailed paired t test).

Inhibition of neuronal uptake (fig. 6.3) or inhibition of MAO (fig. 6.1) had little effect on NMN formation in any group while inhibition of COMT (fig. 6.2) produced a marked increase in DOPEG formation in all treatment groups except the 17β oestradiol treated which increased only slightly.

A) Comparison of control and 17β oestradiol treatments

Was the increase in DOPEG formation due to -

1) increased MAO activity?

2) increased access of NA to neuronal MAO?

3) decreased COMT activity?

4) decreased access of NA to COMT?

Mechanisms 3) and 4) seem unlikely since in homogenates (chapter 4) 17β oestradiol treatment increased uterine COMT activity. Moreover, the increase in DOPEG formation in uterine segments was not accompanied by a decrease in o-methylated metabolites. However it is possible for a decrease in the COMT pathway to increase DOPEG formation (fig. 6.2), but it is not known whether the increase in DOPEG formation in the presence of UO521 is at a neuronal or extraneuronal site. More likely mechanisms for the action of 17β oestradiol on DOPEG formation are 1) and 2). The effect of these three drugs provide no evidence with respect to these two possibilities but an increase in total MAO activity was found in uterine homogenates after 17β oestradiol treatment (chapter 4).

B) Comparison of Progesterone + 17β oestradiol treatment with Controls

DOPEG: Was the decrease in DOPEG formation in the combined steroid groups due to -

1) decreased MAO activity?

2) decreased access of NA to neuronal MAO?

3) increased COMT activity?

4) increased access of NA to COMT?

Mechanisms 1) and 2) are suggested by the effects of cocaine and nialamide on DOPEG formation. However, the data here do not provide a test of these two mechanisms. Total MAO activity increased in the combined steroid treated group relative to controls but it is not known to what extent changes in neuronal MAO paralleled this trend. Mechanism 3) seems possible since COMT activity increased in homogenates from this group (chapter 4). However it also increased to the same extent in 17β oestradiol treated rabbits in which DOPEG formation increased. Mechanisms 3) and 4) are both possible since the difference between the treatment groups disappeared when COMT was inhibited by U0521. Nevertheless, no conclusions can be reached with respect to the effect of progesterone + 17β oestradiol treatment on DOPEG formation.

NMN

Possible mechanisms for the increased NMN formation in the combined steroid treated group relative to controls are -

1) increased COMT activity.

2) increased access of NA to COMT.

3) decreased MAO activity.

4) decreased access of NA to neuronal MAO.

Since neither nialamide nor cocaine, both of which reduced DOPEG formation to low levels, had any effect on NMN formation, mechanisms 3) and 4) are unlikely. Moreover MAO activity of uterine homogenates was increased in the combined steroid group compared with controls. Mechanism 1) is possible but unlikely since, although COMT activity increased in uterine homogenates from the combined steroid treated group, it increased to the same extent

TABLE 6.3. Effect of ovarian steroids on metabolites of ^{3}HINA in rabbit uterine segments.

Series 2.

TREATMENT		TOTAL ^{3}H	NA + DOMA	DOPEG	VMA	NMN	MOPEG	Σ^{3}H TISS.	Σ^{3}H METABS.
Ovariectomized control	Tissue	38.6±15.9	31.6±16.5	1.5±0.5	1.2±0.6	0.9±0.4	0.7±0.06	35.8 (93%)	-
	Medium	-	-	11.0±3.1	1.0±0.6	2.3±0.5	2.6±0.2	-	-
	Total	-	-	12.5±3.5	2.2±0.7	3.2±1.1	3.3±0.1	-	21.2
17β oestradiol	Tissue	11.9±0.6	**6.8±0.6	0.5±0.1	0.7±0.5	1.7±0.6	0.9±0.1	10.7 (90%)	-
	Medium	-	-	3.1±1.1	0.5±0.4	1.8±0.4	1.8±0.3	-	-
	Total	-	-	**3.6±1.5	1.2±0.6	3.5±1.0	2.7±0.5	-	11.0
Progesterone	Tissue	15.1±3.2	**7.7±1.9	0.6±0.1	1.2±0.4	3.8±0.2	1.2±0.1	14.5 (96%)	-
	Medium	-	-	2.9±0.8	1.5±0.9	3.8±0.6	2.7±0.3	-	-
	Total	-	-	**3.8±1.0	2.8±0.8	*7.6±1.1	3.7±0.5	-	17.8
Progesterone + 17β oestradiol	Tissue	12.8±2.4	**5.2±1.7	0.5±0.1	1.3±0.6	3.9±1.0	1.1±0.06	11.9 (92%)	-
	Medium	-	-	2.0±0.6	1.3±0.3	5.2±0.9	3.1±0.2	-	-
	Total	-	-	**2.5±0.6	2.5±0.7	*9.1±2.3	4.5±0.3	-	18.5

Footnotes:
1) values in nmol (g dry wt)$^{-1}$(30 min)$^{-1}$
2) n = 4
3) 2 tailed unpaired t tests *$p < 0.05$ comparisons with controls
4) Rank Sum Test **$p < 0.05$

in the 17β oestradiol traated group. NMN formation was unchanged in the latter group. Mechanism 2) is therefore the most likely explanation for increased NMN formation after progesterone + 17β oestradiol treatment. The possibility of increased COMT activity as a mechanism will be considered further in relation to rabbits treated with progesterone alone.

C) Progesterone + 17β oestradiol treatment compared with 17β oestradiol treatment.

The only further difference when the combined steroid group was compared with the 17β oestradiol treated group rather than controls was a significant increase in MOPEG formation associated with the progesterone treatment. Since MOPEG formation was unaffected by cocaine treatment (fig. 6.3) it appeared to be formed extraneuronally and independently of the activity of the neuronal pathway for inactivating NA. Possible mechanisms for the increase in MOPEG formation include -

1) increased COMT activity.

2) increased MAO activity.

3) increased access of NA to extraneuronal MAO and COMT.

Mechanisms 1) and 2) seem unlikely in view of the fact that compared with the 17β oestradiol treated group, MAO activity of uterine homogenates was less and COMT activity the same in the combined steroid group. It seems more likely that increased MOPEG formation was due to mechanism 3).

(b) Series 2

(i) Ovariectomized Controls

The distribution of metabolites formed in the four treatment groups in series 2 is shown in table 6.3 Since the data for the ovariectomized control group are very variable, individual data for this group are given in table 6.4.

TABLE 6.4. Metabolites of ^{3}HlNA from uteri from ovariectomized control rabbits.

Series 2 individual data.

		NA + DOMA	DOPEG	VMA	NMN	MOPEG	TOTAL ^{3}H TISSUE
Tissue	1	12.5	0.8	0.4	1.3	0.8	19.2
	2	17.0	1.7	2.9	1.8	0.8	28.4
	3	81.0	2.7	1.3	0.2	0.6	86.2
	4	15.9	0.7	0	0.4	0.6	20.8
Medium	1	-	7.9	2.9	3.1	2.7	-
	2	-	15.0	0.02	3.9	2.6	-
	3	-	17.2	1.2	1.0	2.8	-
	4	-	3.8	0	1.2	2.3	-
Total metabolites	1	-	8.7	3.3	4.3	3.5	-
	2	-	16.7	2.9	5.8	3.3	-
	3	-	19.9	2.6	1.1	3.4	-
	4	-	4.5	0	1.7	2.9	-

Footnote : metabolites are expressed in nmol (g dry wt)$^{-1}$(30 min)$^{-1}$.

TABLE 6.5. Distribution of metabolites formed by uterine segments in series 1 and 2 experiments.

		DOPEG	VMA	NMN	MOPEG
Medium	Series 1	38	6	35	22
	Series 2	65	6	14	15
Tissue	Series 1	15	18	45	18
	Series 2	35	26	21	16

Footnote: metabolites are expressed as percentage of total metabolites.

There was a marked difference between the two series in total ^{3}H accumulated by control uteri, those in series 2 accumulating much more ^{3}H than those in series 1. Moreover the absolute rate of formation of DOPEG per g of tissue was much greater for uteri in series 2 than in series 1 so that DOPEG now represented the major metabolite. Table 6.5 compares the relative % of the total for each metabolite fraction. The proportion of VMA and MOPEG in the tissue extracts and in the incubation media were of the same order in the two series. However in both the tissue and the incubation media, series 2 uteri produced proportionately more DOPEG (59% cf. 32%) and less NMN (15% cf. 38%) compared with those in series 1. The reason for the difference in ^{3}H accumulation and DOPEG formation will be discussed subsequently. At this stage, it may be mentioned that the weights of the uteri in series 2 controls were far less than those in series 1 (table 6.6). In the subsequent section the data will be reviewed in relation to whole organ weights.

(ii) Effect of Steroid Treatments

In contrast to series 1, 17β oestradiol treatment significantly reduced DOPEG formation per g of tissue, compared to controls but as in series 1 had no effect on the other metabolites.

Combined 17β oestradiol + progesterone treatment had a similar effect to that in series 1 in that NMN formation was significantly increased while DOPEG formation was significantly reduced compared with control values However, although showing a tendency to decrease below the value for the 17β oestradiol group, DOPEG formation in the combined steroid treated group was not significantly less than in the 17β oestradiol treated group.

TABLE 6.6. Comparisonsof rabbits in series 1 and 2 experiments with respect to uterine weight.

TREATMENT	SERIES 1			SERIES 2		
	BODY WEIGHT	UTERINE WEIGHT	INCUBATED SEGMENT AS % OF TOTAL	BODY WEIGHT	UTERINE WEIGHT	INCUBATED SEGMENT AS % OF TOTAL
Ovariectomized controls	2.7 ± 0.3	1.5 ± 0.3	3.1 ± 0.8	2.6 ± 0.3	0.3 ± 0.1	25.9 ± 10.9
17β oestradiol	2.8 ± 0.1	2.8 ± 0.3	2.4 ± 0.3	2.9 ± 0.2	1.7 ± 0.2	1.5 ± 0.2
Progesterone	-	-	-	3.2 ± 0.1	1.3 ± 0.6	3.1 ± 1.8
Progesterone + 17β oestradiol	2.6 ± 0.2	4.7 ± 0.7	0.9 ± 0.06	2.7 ± 0.1	3.7 ± 0.4	1.1 ± 0.1
Untreated	2.5 ± 0.2	1.9 ± 0.1	2.1 ± 0.2			

Footnotes: 1) Body weight expressed in Kg, uterine weight in g

NB: uterine weight refers to one horn only.

Accordingly 17β oestradiol treatment did not increase DOPEG formation per g tissue over the other treatment groups as it did in series 1. However, when compared to the 17β oestradiol treated rabbits, uteri from the combined 17β oestradiol + progesterone group formed significantly more NMN and MOPEG as in series 1.

The additional treatment in this series, namely progesterone alone, caused a significant increase in NMN formation per g of tissue when compared with 17β oestradiol treated rabbits and control rabbits. DOPEG formation was not significantly different from that in the oestrogen treated uteri but like all other steroid treatments in this series, it significantly reduced DOPEG formation per g below the level in control uteri.

It was intended to pool the data for the incubating media from series 1 and 2. However due to the differences in variance for the two ovariectomized control groups this data could not be pooled.

(iii) Tissue ^{3}H l NA content

A comparison of the estimates of NA and DOMA in tissue extracts obtained by column chromatography and the estimates of NA + DOMA obtained by TLC is shown in table 6.7. The combined fraction contained predominantly NA as might have been predicted from the data in chapter 5 for untreated rabbits. No difference in tissue NA or DOMA was found for the different steroid treatments. In contrast to series 1 experiments, although 17β oestradiol + progesterone treatment lead to slightly lower tissue ^{3}HlNA values, these were not significantly different from those for the 17β oestradiol treated group. However, values for both NA and

TABLE 6.7. Tissue contents of ^{3}H NA and ^{3}H DOMA in rabbit uterine segments estimated by different techniques.

TREATMENT	CHROMATOGRAPHIC METHOD		
	TLC	COLUMN CHROMATOGRAPHY	
	NA + DOMA	NA	DOMA
Ovariectomized controls	31.6 ± 16.5	27.3 ± 13.9	1.1 ± 0.3
17β oestradiol	6.8 ± 0.6	6.2 ± 1.5	0.4 ± 0.1
Progesterone	7.7 ± 1.9	6.0 ± 1.5	0.5 ± 0.1
Progesterone + 1/β oestradiol	5.2 ± 1.7	4.8 ± 1.7	0.3 ± 0.1

Footnotes: 1) metabolites are expressed in nmol (g dry wt)$^{-1}$(30 min)$^{-1}$.

2) values were obtained from series 2 experiments.

3) n = 4

DOMA were larger and more variable in tissue extracts of control uteri than in those from steroid treated rabbits consistent with the large and variable ^{3}H accumulated by uteri from this group.

(iv) Comparison of Wash Periods

Table 6.8 compares the total ^{3}H accumulated by rabbit uterine segments in series 1 and 2. ^{3}H lost in the 1 minute wash in series 1 represented 12% of the total ^{3}H accumulated in 30 min of incubation. There was a tendency for more ^{3}H to be lost in the 1 min wash solution from uteri in the combined steroid treated group but this trend was not significant. The difference in ^{3}H accumulation by series 1 and 2 control uteri did not appear to be due to differences in wash times since the difference in ^{3}H accumulated vastly exceeded the amount of ^{3}H found in 1.0minute wash solutions. Due to the large SE associated with ^{3}H accumulation in the 17β oestradiol treated group, series 1 and the 17β oestradiol + progesterone treated group, series 2, no further conclusions could be drawn as to the effect of different wash times in these two groups. However, a comparison of the distribution of metabolites in the tissue from these two treatment groups in series 1 and 2 indicated that they were not influenced by the wash period since the metabolites were comparable in both series (table 6.1 cf table 6.3).

(v) Amine in the Extracellular Space

Table 6.9 compares the estimated equivalent amount of ^{3}HINA present in the extracellular space of uteri from the different treatment groups as estimated by ^{14}C sorbitol present in tissue extracts. There was no significant difference between treatment groups although there was a tendency for this parameter to decrease in tissues from 17β oestradiol

TABLE 6.8. Effect of wash period on total ^{3}H in uterine tissues.

TREATMENT	1.0 MIN WASH (SERIES 1)			10 SEC WASH (SERIES 2)
	TOTAL ^{3}H IN TISSUE	^{3}H IN WASH	^{3}H IN TISSUE & WASH	TOTAL ^{3}H IN TISSUE
Ovariectomized controls	**12.7±1.2	1.9±0.5	14.6±1.3	38.6±15.9
17β oestradiol	10.6±3.2	1.6±0.4	12.2±3.0	*11.9± 0.6
Progesterone	-	-	-	*15.1±3.2
Progesterone + 17β oestradiol	13.6±0.7	2.5±0.3	16.1±0.5	*12.8±2.4

Footnotes: 1) n = 5 series 1
n = 4 series 2

2) metabolites are expressed in nmol (g dry wt)$^{-1}$(30 min)$^{-1}$

3) * $p < 0.05$ Rank Sum Test
** $p < 0.03$ Rank Sum Test

Differences were tested relative to series 2 controls.

TABLE 6.9. Equivalent amount of NA estimated to be in the uterine extracellular space.

TREATMENT	NA (nmol g^{-1})
Control	4.5 ± 0.5
17β oestradiol	3.9 ± 0.7
Progesterone	5.6 ± 0.9
Progesterone + 17β oestradiol	5.0 ± 0.8

Footnotes: 1) n = 4

2) estimates were made by incubating segments with INA and ^{14}C sorbitol for 30 min and washing for 10 secs in amine-free Krebs solution.

treated animals and to increase in those from progesterone treated uteri. In the first series of experiments in which tissues were washed for 1.0 min no estimates of the amount of amine in the extracellular space were made.

(c) Formation of Metabolites per Uterine Horn

(i) Differences between Controls

Since there were marked differences in the uterine weights of the ovariectomized rabbits in the two series of experiments (table 6.6) the data on metabolite formation in tables 6.1 and 6.3 have been re-expressed per uterine horn in table 6.10. When expressed in this way the formation of DOPEG, NMN, MOPEG and VMA per uterine horn is less in series 2 controls than in series 1, as is the tissue content of NA + DOMA. The discrepency between tissue NA + DOMA and DOPEG formation for the two series is somewhat less marked but still apparent (3 fold cf 4 fold for NA + DOMA and 2.5 fold cf 3 fold for DOPEG). In contrast, the formation of the remaining metabolites by the whole uterine horn is much less in series 2 controls since their formation on a weight basis was similar in the two series but means uteri from series 2 is only 20% of that in series 1.

Since endogenous NA concentration was shown to be inversely proportional to uterine weight in ovariectomized control animals in chapter 4, one might expect series 2 controls to have a lower endogenous NA concentration than series 1.controls. Thus for the same total number of sympathetic nerves per uterine horn, the density of innervation would be greatest for series 2 controls thereby leading to a greater accumulation of ^{3}HlNA and greater formation of DOPEG. Unfortunately endogenous NA

TABLE 6.10. Metabolites of ^{3}HINA formed by rabbit uteri expressed per uterine horn.

TREATMENT	SERIES 1					SERIES 2				
	TISSUE NA + DOMA	TOTAL METABOLITES				TISSUE NA + DOMA	TOTAL METABOLITES			
		DOPEG	VMA	NMN	MOPEG		DOPEG	VMA	NMN	MOPEG
Control	2.0±0.7	1.1±0.4	0.4±0.2	1.4±0.7	0.9±0.5	0.7±0.2	0.4±0.2	0.1±0.06	0.2±0.1	0.1±0.06
17β oestradiol	1.8±0.4	*1.8±0.3	0.4±0.1	0.9±0.3	0.6±0.1	1.1±0.1	0.5±0.2	0.2±0.1	*0.5±0.1	0.4±0.06
Progesterone	N.D.	N.D.	N.D.	N.D.	N.D.	1.1±0.4	0.7±0.4	*0.5±0.3	**1.1±0.5	**0.9±0.4
Progesterone + 17β oestradiol	1.8±0.3	0.7±0.3	0.7±0.2	*4.2±0.9	1.6±0.2	1.7±0.7	0.7±0.2	**0.8±0.2	***2.8±0.7	***1.4±0.2

Footnotes: 1) metabolites are expressed as nmol (uterine horn)$^{-1}$(30 min)$^{-1}$.

2) n = 4 series 2, n = 5 series 1.

3) unpaired, 2-tailed t tests compared with controls * $p < 0.05$
** $p < 0.01$
*** $p < 0.005$

contents were measured only for series 2 controls. Moreover the lower formation of DOPEG per uterine horn in series 2 controls suggest that they may have less sympathetic nerves per uterine horn than series 1 even though their density of innervation is probably greater due to the 5 fold difference in mean uterine weight for the two series.

(ii) Effect of Steroid Treatments

The effect of progesterone in combination with 17β oestradiol was to significantly increase the o-methylating capacity of the uterus in both series 1 and 2 whether expressed on a weight basis or per uterine horn. In table 6.10 this is evident by the significant increase in NMN and MOPEG formation per uterine horn when compared to controls in both series 1 and 2. In series 2 it also significantly increased VMA formation per uterine horn. Progesterone treatment by itself also significantly increased the o-methylating capacity of the uterus in that VMA, NMN and MOPEG formation per uterine horn were significantly increased with respect to controls. NMN formation in progesterone treated uteri was also significantly greater than in 17β oestradiol treated uteri.

DOPEG formation per uterine horn was not reduced compared with controls in progesterone treated rabbits, or those treated with progesterone + 17β oestradiol.

The effect of 17β oestradiol treatment at this stage must be regarded as equivocal. DOPEG formation per whole uterine horn as well as per g was significantly increased by this treatment in series 1 when compared to controls or to uteri from 17β oestradiol + progesterone treated uteri but this was not the case in series 2. In series 2, the

decreased formation of DOPEG per g of uterine tissue when compared to controls appeared to be the result of 17β oestradiol on uterine size since DOPEG formation per uterine horn was comparable in the controls and 17β oestradiol group. In series 1, 17β oestradiol had no effect on the o-methylating capacity of the uterus as a whole but in series 2, due to its effect on uterine size it significantly increased NMN formation per uterine horn compared with series 2 controls but not when compared with series 1 controls.

(d) Relationship between endogenous NA concentration and ^{3}H l NA accumulation and DOPEG formation

The correlation between these variables was estimated to assess whether the ^{3}H l NA content of the tissue had a relation to the density of sympathetic innervation as assessed by endogenous NA concentration. DOPEG formation was also included in view of its apparent neuronal origin. This data was derived from series 2 experiments. As shown in fig. 6.4 there is a good correlation between the accumulation of ^{3}H l NA per g of tissue and endogenous NA concentration. There is also a correlation between endogenous NA concentrations and DOPEG formation although less significant.

These results accord with an assumption, based on the effects of cocaine that a major part of the ^{3}H l NA in the tissue and of DOPEG formation is neuronal in location. It should be noted that the data includes the 17β oestradiol + progesterone treated group although it had been shown that the ^{3}H l NA taken up by the tissue was relatively cocaine - insensitive in this group. However the ^{3}H l NA levels were lowest in this group.

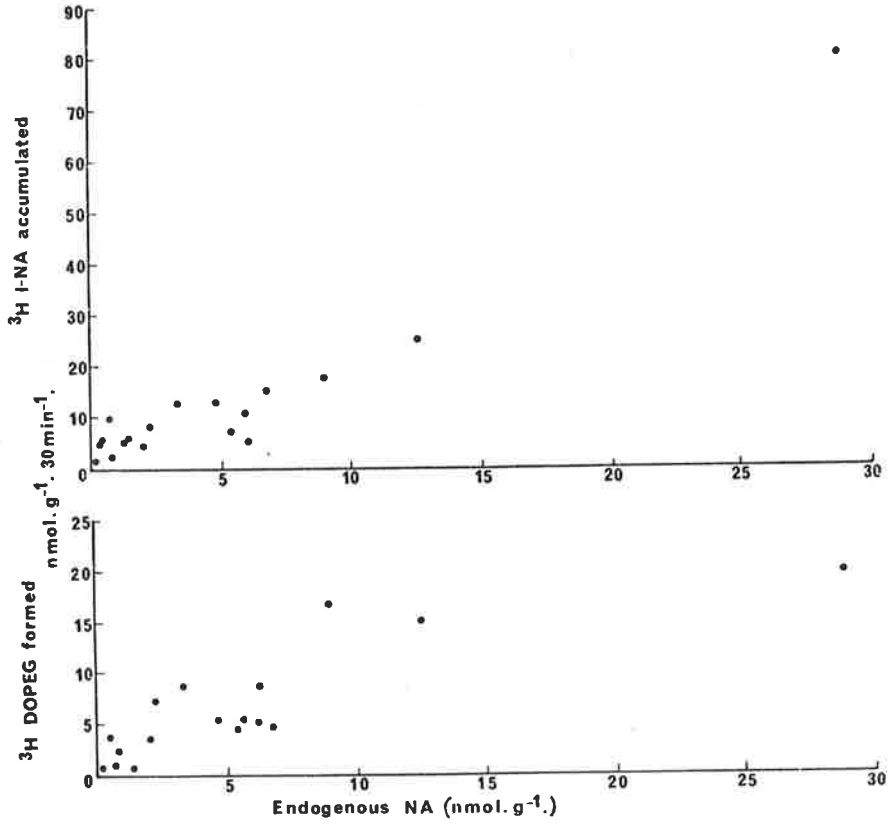

Fig. 6.4 Relationship between the endogenous NA concentration of the rabbit uterus and (A) the ^{3}H l NA accumulated by and (B) ^{3}H DOPEG formed in uterine segments. A. correlation coefficient = 0.96 ($p < 0.001$) B. correlation coefficient = 0.81 ($p < 0.05$).

DISCUSSION

1. EFFECT OF UTERINE SIZE

The positive correlation between endogenous NA concentration and ^{3}HINA accumulation and DOPEG formation when expressed per g of uterine tissue was evident in series 2 experiments (fig. 6.4) in that the smaller ovariectomized control uteri were associated with larger amounts of ^{3}HINA and DOPEG. Smaller uteri would be expected to possess larger concentrations of endogenous NA for a given content of NA per uterus since in chapter 4 these two variables were shown to be inversely related. If the amount of DOPEG formed and ^{3}HINA accumulated were expressed per uterine horn the correlation should disappear, as was in fact the case, no difference in these two variables being apparent between control uteri and the larger uteri from steroid treated rabbits. However it was possible that series 1 and 2 controls differed in the total number of nerves per uterine horn since ^{3}HINA accumulation and DOPEG formation per uterus horn were lower in series 2 controls. Unfortunately the critical measurement to test this suggestion, namely that of endogenous NA content, was not performed in series 1.

2. EFFECT OF STEROID TREATMENTS

To assist discussion the results in this chapter are further summarized qualitatively in table 6.11. The effects of the various treatments on organ weight, ^{3}HINA accumulation, metabolite formations and enzyme activity are summarized relative to ovariectomized control rabbits.

TABLE 6.11. Summary of effects of steroid treatments on rabbit uteri.

	UNTREATED	17β OESTRADIOL	PROGESTERONE	PROGESTERONE + 17β OESTRADIOL
Uterine weight	↑	↑	N.S. (↑)	↑ ↑
MAO/g protein	↔	↑ ↑	↑	↑
MAO/uterus	↑	↑ ↑	↑	↑ ↑
COMT/g protein	↔	↑	↔	↑
COMT/uterus	↑	↑ ↑	↔	↑↑↑
NA/g	↓	↓	N.S. (↓)	↓
NA/uterus	↔	↔	↔	↔
	series 1 series 2	1 2	1 2	1 2
^{3}HINA/g	↔ ↓	↔ ↓	- ↓	↓ ↓
^{3}HINA/uterus	↔ ↔	↔ ↔	- ↔	↔ ↔
^{3}H metabs. (nmol/g)				
DOPEG	↑	↑ ↓	- ↓	↓ ↓
DOMA	-	- ↓	- ↓	- ↓
MOPEG	↑	↔ ↔	- ↔	↔ ↔
NMN	↔	↔ ↔	- ↑	↑ ↑
VMA	↔	↔ ↔	- ↔	↔ ↔
(nmol/uterus)				
DOPEG	-	↑ ↔	- ↔	↔ ↔
DOMA	-	- ↔	- ↔	- ↔
MOPEG	-	↔ ↑	- ↑	↑ ↑ ↑
NMN	-	↔ ↑	- ↑ ↑	↑ ↑↑↑
VMA	-	↔ ↔	- ↔	↔ ↑

Footnotes: 1) all changes are related to ovariectomized controls.

2) N.S. = not significant; - = not tested; ↑ = increase; ↓ = decrease; ↔ = no change

(a) ^{3}HlNA accumulated

The steroid treatments had no significant effect on the amount of ^{3}HlNA accumulated per uterine horn. However per g of uterine tissue all steroid treatments decreased the amount of ^{3}HlNA accumulated by uterine segments when compared to those from ovariectomized control animals in series 2. This effect appeared to be due to the relatively low endogenous NA concentration of uteri from the steroid treated groups when compared with those from series 2 controls indicating a lower density of sympathetic nerves. In series 1, ^{3}HlNA accumulated per g was lower in the combined steroid treated group than controls but unaltered by 17β oestradiol treatment. It should be noted that although ^{3}HlNA accumulated per uterine horn in the combined steroid treated groups did not differ significantly from that in the other treatment groups it did appear to have a different location in the tissue. Most appeared to be located at extraneuronal sites since it was relatively insensitive to cocaine.

(b) Metabolites of ^{3}HlNA

(i) 17β oestradiol

The effect of 17β oestradiol pretreatment appears equivocal in that in series 1, it increased DOPEG formation, both per g of tissue and per whole uterine horn whereas in the second series it had no effect on total DOPEG formation per uterine horn and, in fact, decreased DOPEG formation per g of tissue relative to the control uteri. Part of the discrepancy can be attributed to the tendency of this steroid to increase uterine weight without increasing the number of sympathetic nerves to the same degree as indicated by a decline in endogenous NA concentration (see chapter 4).

In the second series, the endogenous NA content of the whole uterine horn did not decrease after 17β oestradiol treatment but the marked decrease on a weight basis due to the large difference in uterine weight apparently accounted for the decreased formation of DOPEG per g of tissue. In series 1 the difference in uterine weight between control and 17β oestradiol treated rabbits was much less marked and no decline in DOPEG formation occurred.

However the discrepancy still remains that DOPEG formation increased in the first series, even when expressed on a total organ basis. This result implies that oestradiol treatment may have, as a second effect, a tendency to enhance oxidative deamination per unit density of sympathetic nerves. This is consistent with the earlier finding that 17β oestradiol treatment increased MAO activity per mg of protein and per uterine horn (table 4). However it has not been established whether intraneuronal MAO activity increases in a similar way. Whatever the cause, there is, as yet, no obvious explanation why this effect did not also occur in the second series of experiments. There is also some indication that uteri from untreated rabbits produced more DOPEG per g tissue than those of similar size from ovariectomized control animals (table 5). One possible reason is the reduced formation of oestrogen in the ovariectomized animals.

(ii) Progesterone

The effect of progesterone on the uterus appears more straightforward than that of 17β oestradiol. Progesterone, either alone or in combination with 17β oestradiol, increased the formation of extraneuronal o-methylated metabolites, both per g of tissue and per uterine horn. This appeared to

be due to an increase in access of the substrate to extraneuronal COMT since progesterone by itself did not increase COMT activity in homogenates (chapter 4). Moreover increased access to extraneuronal COMT does not appear to result indirectly from decreased access of NA to MAO, either neuronal or extraneuronal, since neither nialamide nor cocaine, which reduced DOPEG formation to very low levels, had any effect on NMN formation in this tissue. At first sight the increase in o-methylation may be viewed as a simple consequence of growth of extraneuronal tissues ie smooth muscle relative to sympathetic nerves produced by steroid treatment. Thus, in both series, the combined steroid treatment, 17β oestradiol + progesterone, caused the greatest increase in uterine weight, the greatest decrease in endogenous NA concentration in the tissue and the greatest increase in NMN formation per g of tissue. This meant also that the o-methylating capacity of the whole uterus was increased by all treatments causing increased growth of the uterus. However, the presence of progesterone itself seems essential to the increase in the o-methylating capacity of the uterus per g of tissue since -

1) 17β oestradiol which increased uterine weight to a greater extent than progesterone alone and decreased NA concentration of the uterus to a greater extent than progesterone alone did not increase the formation of o-methylated metabolites per g of tissue. Moreover, even when DOPEG formation was inhibited by cocaine, uteri from 17β oestradiol treated rabbits did not form more NMN than controls.

2) It should also be noted that although progesterone treatment by itself tended to increase uterine weight relative to series 2 controls this effect was not significant. Nevertheless, it caused a significant increase in NMN formation per g of tissue.

In summary, certain possible mechanisms of progesterone's effect on o-methylation can be excluded viz increased COMT activity and decreased neuronal inactivation; however a positive mechanism still remains to be established.

The decrease in DOPEG formation per g of tissue caused by 17β oestradiol + progesterone treatment appeared to be due to the stimulatory effect of the combined treatment on uterine growth since DOPEG formation per uterine horn was unaffected by this treatment. Nevertheless, it is still possible that extraneuronal o-methylation may limit neuronal inactivation of NA in the uterus. This has not been tested directly but inhibition of COMT causes a marked increase in DOPEG formation. Whether the increased DOPEG formed in the presence of UO521 results from the activity of neuronal or extraneuronal MAO is not known and might be tested by the use of cocaine and UO521 simultaneously.

An examination of the kinetics of uptake of NA into neuronal and extraneuronal compartments in the uterus should provide further necessary information on the effect of these steroids on access of NA to intracellular sites of MAO and COMT. Such an approach might help to resolve some of the questions posed by the effect of these steroids on metabolite formation. Studies of the uptake of ISO in the rat heart have provided critical information on extraneuronal compartments for the accumulation of catecholamines (Trendelenburg, 1976) and ISO might also be successfully employed for this purpose in the uterus.

There was no evidence of an inhibitory action on extraneuronal accumulation of NA by the uterus caused by pretreatment with oestrogen and progesterone in this study. However in light of their inhibitory effect on extraneuronal uptake in the rat heart (Iversen & Salt 1970) it is necessary to establish their acute effect on extraneuronal uptake in the uterus.

3. COMPARISON WITH OTHER TISSUES

(a) Non Reproductive Tissues

The comparison with ear arteries in the present experiments indicates that the effect of oestrogen and progesterone on metabolism of ^{3}HlNA was relatively specific for the uterus. Thus in series 1 experiments the treatments were without effect on the pattern of metabolite formation and distribution in ear arteries from the same rabbits. The tissue levels of ^{3}H and ^{3}HlNA reported here for rabbit ear arteries compare well with those reported by Head (1976) for untreated rabbits. Similarly DOPEG was the major metabolite in ear arteries (70%) although the amount reported by Head in tissue extracts was higher (approx. twice).

The difference between the effect of these steroids on the two tissues may be due to a number of factors. Firstly the ear artery, as a non-target tissue, is not subject to changes in weight and tissue composition as is the uterus and does not undergo changes in its relative density of innervation (see chapter 4). Secondly there might be large differences in the local tissue concentration of the steroids in the two tissues for a given circulating plasma concentration. Bell & Malcolm (1978) have explained a selective effect of progesterone on guinea pig

uterine catecholamine fluorescence in this way since implantation of progesterone in the gastrocnemius muscle of the guinea pig caused a similar loss of fluorescence associated with the blood vessels in this area. In the present case, the rabbit uterus has specific receptors for the ovarian steroids (ElBanner & Sacher 1977) and would be expected to have higher tissue concentrations than the ear artery. However it is not known whether the steroids accumulated in the uterus in this way have access to the catecholamine uptake processes and metabolizing enzymes.

(b) Reproductive Tissues

Green and Miller (1966a) showed a negative correlation between uterine weight and accumulation per gram of ^{3}H NA and ^{3}H A by rat uterine slices *in vitro*. The amount of catecholamine accumulated by the whole uterus was not correlated with organ weight. One possible explanation offered by them was a declining density of innervation with increasing weight of the uterus. One might have expected such a correlation to be less marked in the rat uterus which has a low density of innervation (Norberg & Fredriccson 1966). However most of the catecholamine accumulated by their uterine slices appeared to be taken up by nerves since it was reduced by 90% by cocaine. The predominance of neuronal accumulation of catecholamines may reflect the substrate concentration used by these authors, the concentration being 1/40 of that used in the present study. Although Green and Miller showed that 17β oestradiol treatment increased uterine weight and decreased the accumulation of ^{3}H l NA as in series 2 of the present study, they were unable to detect metabolites of NA so that comparisons of changes in metabolites with the present study are not possible.

4. SITES OF FORMATION OF METABOLITES IN THE UTERUS

A complicating factor is that it is not known which of the various components of the uterine tissue (endometrium, myometrium, blood vessels) contribute to the formation of the metabolites measured in the present experiments. Presumably the production of DOPEG (mainly neuronal) is associated with nerve cells both between the myometrial smooth muscle cells and around the blood vessels between the circular and longitudinal muscle layers of the myometrium. Since uterine segments were not subdivided the role of the endometrium in metabolism of NA by the uterus has not been established. It is known that the endometrium in the human displays MAO activity (Collins *et al.*, 1969). Further experiments on isolated endometrium and myometrium may help to establish the relative contributions of these two regions to the inactivation of NA in the rabbit uterus.

CHAPTER 7

EFFECT OF PREGNANCY ON THE METABOLISM OF ^{3}HINA BY RABBIT UTERINE SEGMENTS

INTRODUCTION

In chapter 6, the results of pretreatment of rabbitswith oestrogen and progesterone for 14 days indicated that their combined action resulted in increased extraneuronal and decreased neuronal metabolism of NA. Probable reasons for this effect were the stimulatory effect of progesterone on NMN formation and the stimulatory effect of the combined treatment on uterine growth, resulting effectively in a decreased density of innervation. The present experiments were designed to test whether similar changes in metabolism of NA by the rabbit uterus occur in pregnancy.

In the rabbit, oestrogen and progesterone secretion by the ovary increases during pregnancy and has been estimated to peak at 8-10 days (20.5 ng/ovary/hr) for 17β oestradiol and at 14-18 days (78.3 µg/ovary/hr) for progesterone (Hilliard & Eaton 1971, Hilliard *et al.*, 1973). The equivalent level of 17β oestradiol and progesterone in peripheral venous plasma at these times were 224 pg/ml and 12 ng/ml respectively. For comparison the treatment in chapter 6 achieved similar plasma levels for progesterone viz 9.6 ng/ml but oestradiol levels were not detected by an assay in which the limit of sensitivity was 20 pg/ml. In the rabbit, progesterone levels begin to decline approximately 26 days after mating (Hilliard *et al.*, 1973) to reach low levels at day 30-32. Additional secretion of oestrogen and progesterone is afforded by the developing foeto-placental unit but in the rabbit, the ovary is a major source of steroids and is necessary for the maintenance of pregnancy throughout the gestation period (Keyes and Nalbandov 1967).

The rabbits were used in late pregnancy, 28 days after mating, at a time when progesterone levels are still elevated. Segments of uteri were prepared from implantation as well as non-implantation sitessince it was anticipated that the specialization of tissues at the implantation site might produce a different pattern of metabolites.

METHODS

Rabbits were mated in individual cages and the day of mating recorded. Pregnant rabbits were killed 28 days after mating, at which time the mean number of foetuses was recorded (7 ± 1; $n = 6$). 40 mg segments, 1.0 mm in length were prepared as described in chapter 3 with the exception that complete rings of uterus were not used because of the large diameter of the pregnant uterus. Segments were prepared from both implantation and non-implantation sites in each rabbit. Ear arteries, ovaries and oviducts were also removed from each rabbit and prepared as described in chapter 2. The tissues were incubated with ^{3}HINA as described in chapter 3. They were washed for 1.0 min in amine free Krebs after incubation and then extracted for ^{3}H metabolites. Uterine segments from each rabbit were also incubated in the presence of cocaine (29 μmol l^{-1}) as described in chapter 5.

RESULTS

1. PREGNANT UTERUS

The distribution of the metabolites in the tissue and in the incubating medium of pregnant uteri are compared with those in non-pregnant uteri in table 7.1 and fig. 7.1. The data on the pregnant uteri are divided according to the location of the segment i.e. whether excised from implantation sites or between implantation sites. It will

TABLE 7.1. Effect of pregnancy on the metabolism of ^{3}HINA by rabbit uterine segments

TREATMENT		TOTAL ^{3}H	NA + DOMA	DOPEG	VMA	NMN	MOPEG	Σ^{3}H TISS.	Σ^{3}H METABS.
Non pregnant (n = 10)	Tissue	17.3±2.7	9.3±1.9	1.4±0.2	1.7±0.5	1.2±0.1	1.2±0.1	14.8 (86%)	5.5
	Medium	-	-	6.7±1.8	0.6±0.2	2.7±0.4	4.6±0.6	-	14.6
	Total	-	-	8.1±1.6	2.3±0.5	3.9±0.4	5.8±0.6	-	20.1
Pregnant implantation site (n = 5)	Tissue	17.9±1.4	5.6±0.7	0.3±0.06	1.4±0.3	3.9±0.5	0.9±0.3	12.0 (67%)	6.4
	Medium	-	-	1.5±0.3	1.0±0.3	6.8±1.2	2.0±0.5	-	11.3
	Total	-	-	1.8±0.3	2.4±0.6	10.7±1.5	**2.8±0.4	-	17.7
Pregnant non-implantation site (n = 6)	Tissue	19.4±2.0	**5.3±0.7	0.3±0.06	1.4±0.2	6.9±1.2	1.2±0.2	14.2 (73%)	9.8
	Medium	-	-	2.2±0.5	0.8±0.3	12.3±2.7	3.1±0.5	-	18.4
	Total	-	-	*2.5±0.4	2.3±0.5	*19.2±3.5	4.2±0.3	-	25.4
Pregnant non-implantation site + cocaine (n = 5)	Tissue	13.8±1.8	4.3±1.4	0.1±0.02	1.6±0.3	5.4±0.7	1.0±0.1	12.3 (89%)	8.0
	Medium	-	-	0.9±0.01	0.7±0.3	9.5±2.3	2.3±0.4	-	12.5
	Total	-	-	***1.0±0.01	2.2±0.1	14.9±2.6	3.3±0.5	-	20.5

Footnotes: 1) metabolites are expressed in nmol (g dry wt)$^{-1}$(30 min)$^{-1}$.

2) Rank Sum Test * $p < 0.05$ } compared with controls
unpaired t test ** $p < 0.05$ }
*** $p < 0.05$ compared with pregnant uteri

be seen that the tissue levels of the NA + DOMA fraction, and the tissue + medium levels of DOPEG and VMA were of the same order irrespective of the site of removal of the segment. However, the tissue + medium levels of NMN and MOPEG were greater in the uterine segments from the non-implantation sites. Nevertheless, with both types of segment, the metabolite distribution was characterized by the marked predominance of NMN, representing 60-76% of total metabolites; indeed in the case of the tissue from the non-implantation site the level of NMN exceeded that of the NA + DOMA fraction. Comparisons with a further five segments from non-implantation sites (table 7.1) showed that cocaine caused a significant decrease in DOPEG formation but did not cause significant changes in the formation of the remaining metabolites. However the decrease in DOPEG formation caused by cocaine was only 60% i.e. its effect was less than that observed previously in untreated uterine segments. These results indicated that a considerable proportion of the DOPEG formation and all the NMN, VMA and MOPEG were formed extraneuronally in the pregnant uterus. The effect of cocaine on tissue levels of the NA + DOMA fraction was small (18%) and not significant on a paired t test, suggesting also that most of this fraction was extraneuronal in distribution.

2. COMPARISONS WITH NON-PREGNANT UTERUS

The data on non-pregnant uteri from an earlier table (table 5.2) are included in table 7.1 for comparison with pregnant uteri. The most striking difference between the metabolite distributions is represented by the 2.5 to 5 fold greater levels of NMN and 3 fold smaller levels of DOPEG in the pregnant uteri. Tissue levels of the NA + DOMA fraction were also 43% lower in pregnant uteri than in non-pregnant uteri.

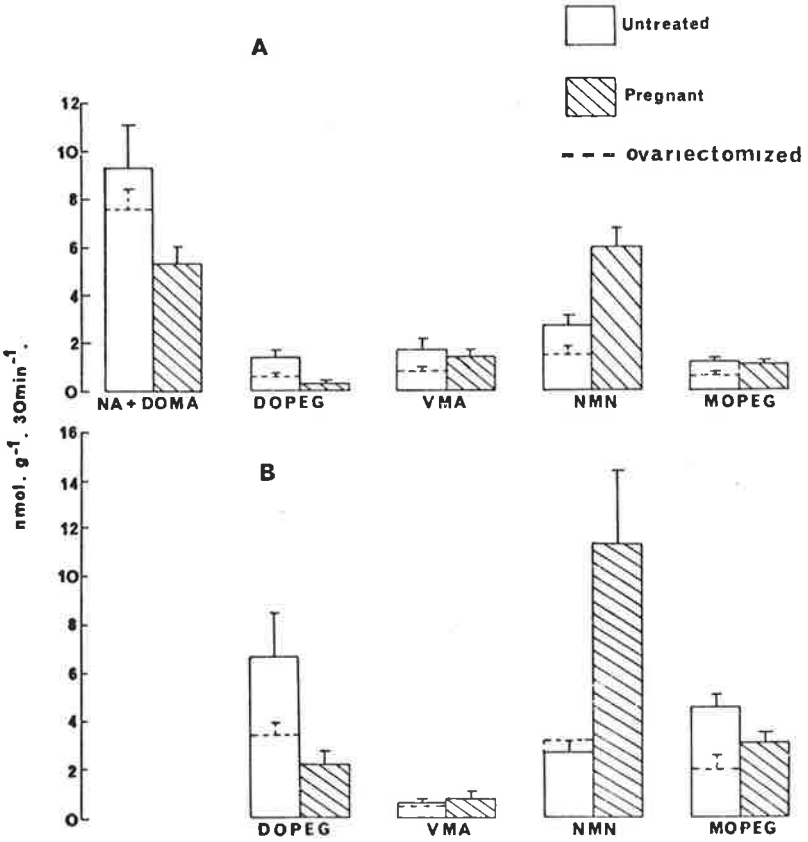

Fig. 7.1 The effect of pregnancy on the metabolites of ^{3}H l NA formed by rabbit uteri. A = tissue contents, B = incubating medium contents of metabolites. Values are means ± SEs of 6 experiments for pregnant rabbits and 10 experiments for untreated, non-pregnant rabbits.

These differences are very similar to those between 17β oestradiol + progesterone treated uteri and uteri from ovariectomized control animals in table 6.1 of the preceding chapter. Fig. 7.1 includes a comparison between pregnant uteri and those from ovariectomized controls. The increase in NMN formation is more marked, although the difference in DOPEG formation is less marked (and no longer significant) when compared with ovariectomized controls rather than untreated uteri.

3. COMPARISONS BASED ON WHOLE UTERI

Since the weights of uteri from the pregnant animals were greater than those of the non-pregnant rabbits by a factor of 10 (n = 6) the difference between the rates of formation of NMN in pregnant and non-pregnant uteri were even more marked (43 fold) when the data was expressed as the amount of metabolites formed per uterine horn.

However the amount of all the other metabolites formed per uterine horn as well as the total tissue content of the NA + DOMA fraction were also greater in the pregnant animals (table 7.2). These results are compatible with the proposition that the increase in the size of the uterus is associated with a corresponding marked increase in the size of the extraneuronal uptake and metabolizing compartment which is not associated with a corresponding increase in the size of the neuronal compartment. However a small increase in the size of the neuronal compartment cannot be excluded if it is assumed that the cocaine-sensitive component of DOPEG formation is an indication of the size of the neuronal compartment. It can be calculated (table 7.2) that the total amount of cocaine-sensitive DOPEG which is formed in the whole pregnant uterine horn is approximately twice that in the non-pregnant uterus.

TABLE 7.2. Effect of pregnancy on formation of metabolites of ^{3}HlNA per uterine horn.

TREATMENT	TISSUE CONTENT OF NA + DOMA	TOTAL METABOLITES				MEAN UTERINE WEIGHT
		DOPEG	VMA	NMN	MOPEG	
Untreated (n = 10)	1.8 ± 0.2 (1.1 ± 0.1)	1.3 ± 0.2 (1.0 ± 0.2)	0.5 ± 0.1	0.7 ± 0.1	1.1 ± 0.1	1.9 ± 0.1
Pregnant (n = 6)	10.5 ± 2.6 (1.8 ± 0.5)	3.5 ± 0.6 (2.1 ± 0.5)	3.7 ± 0.9	31.0 ± 4.8	6.5 ± 0.4	20.2 ± 0.9

Footnotes: 1) metabolites are expressed as nmol (uterine horn)$^{-1}$(30 min)$^{-1}$ uterine weight is expressed in g and refers to one horn only.

2) figures in brackets refer to the amounts sensitive to cocaine.

3) values for pregnant uteri refer to non-implantation sites.

4. COMPARISON WITH OTHER TISSUES

(a) Ovary

Pregnancy had a marked effect on the uptake and metabolism of ^{3}HlNA in the ovary compared with that in untreated rabbits (table 7.3). The tissue content of the NA + DOMA fraction was markedly decreased as was the formation of all of the metabolites. The decreases amounted to 1) NA + DOMA 86% 2) DOPEG 84% 3) VMA 68% 4) NMN 73% and 5) MOPEG 73%. However the relative proportions of the metabolites were the same in pregnant and non-pregnant animals being DOPEG 18%, VMA 17%, NMN 58% and MOPEG 7% for ovaries from pregnant rabbits and DOPEG 18%, VMA 11%, NMN 52% and MOPEG 9% for ovaries fron non-pregnant rabbits. It appeared from these results that the uptake and metabolizing capacity of the ovary per unit weight of tissue was substantially reduced by pregnancy.

(b) Oviduct

In contrast, the accumulation and metabolism of ^{3}HlNA appeared to be the same in oviducts from pregnant and non-pregnant rabbits (table 7.3).

(c) Ear Arteries

The data in table 7.4 indicated that the metabolism of ^{3}HlNA in ear arteries from pregnant rabbits was not significantly different from that in ovariectomized control animals. For comparison the same data from Head (1976) in arteries from untreated rabbits is included. Unfortunately only data for tissue contents is available from his study but it does not indicate any major difference between the untreated arteries and the two other groups of arteries shown in table 7.4.

TABLE 7.3. Effect of pregnancy on the metabolism of ^{3}HINA by rabbit ovaries and oviducts

TISSUE	TREATMENT		TOTAL ^{3}H	NA + DOMA	DOPEG	VMA	NMN	MOPEG	Σ^{3}H TISSUE	Σ^{3}H METABS.
Ovary	Non-pregnant (n = 4)	T	9.4±3.3	4.4±1.7	0.4±0.1	0.8±0.2	1.5±0.5	0.4±0.1	7.5 (80%)	-
		M	-	-	2.5±0.8	0.2±0.1	3.8±1.3	0.7±0.2	-	-
		TOTAL	-	-	2.9±0.9	1.1±0.3	5.3±1.8	1.1±0.3	-	10.2
	Pregnant (n = 4)	T	*2.0±0.3	*0.6±0.1	0.1±0.004	0.1±0.03	0.4±0.1	0.1±0.02	1.4 (70%)	-
		M	-	-	0.4±0.1	0.2±0.1	1.0±0.3	0.2±0.04	-	-
		TOTAL	-	-	*0.5±0.1	*0.3±0.1	*1.5±0.4	*0.3±0.1	-	2.6
Oviduct	Non-pregnant (n = 4)	T	8.9±1.5	6.8±1.1	0.4±0.1	0.6±0.2	0.1±0.01	0.3±0.03	8.1 (91%)	-
		M	-	-	6.0±1.1	0.3±0.04	0.4±0.02	0.9±0.04	-	-
		TOTAL	-	-	6.4±1.1	0.8±0.3	0.5±0.02	1.2±0.03	-	8.9
	Pregnant (n = 4)	T	9.0±1.4	5.8±0.6	0.4±0.1	0.3±0.04	0.1±0.03	0.2±0.05	6.8 (80%)	-
		M	-	-	4.3±0.9	0.6±0.1	0.4±0.1	0.6±0.1	-	-
		TOTAL	-	-	4.7±1.0	0.8±0.2	0.5±0.1	0.8±0.2	-	6.8

Footnotes: 1) metabolites are expressed in nmol (g dry wt)$^{-1}$(30 min)$^{-1}$

2) m = medium; t = tissue

3) unpaired t test * $p < 0.05$

TABLE 7.4. Effect of pregnancy on metabolism of ^{3}HlNA by rabbit ear arteries.

TREATMENT		TOTAL ^{3}H	NA + DOMA	DOPEG	VMA	NMN	MOPEG	Σ^{3}H TISSUE
Pregnant (n = 6)	Tissue	5.5±0.7	4.0±0.3	0.3±0.2	0.03±0.02	0.04±0.01	0.04±0.01	4.4 (80%)
	Medium	-	-	1.6±0.3	0.06±0.05	0.2 ±0.05	0.3 ±0.1	-
	Total	-	-	1.9±0.4	0.08±0.05	0.3 ±0.06	0.3 ±0.1	-
Ovariectomized Control (n = 5)	Tissue	5.6±1.5	4.5±0.2	0.2±0.1	0.07±0.03	0.07±0.03	0.03±0.01	4.8 (87%)
	Medium	-	-	1.9±0.6	0.3 ±0.2	0.3 ±0.1	0.2 ±0.06	-
	Total	-	-	2.1±0.7	0.3 ±0.2	0.4 ±0.2	0.2 ±0.1	-
Untreated (Head 1976)	Tissue	8.2±0.5	4.3±0.5	0.4±0.1	0.1 ±0.04	0.1 ±0.02	0.03±0.01	4.9 (60%)

Footnote : Metabolites are expressed as nmol (g wet wt)$^{-1}$(30 min)$^{-1}$

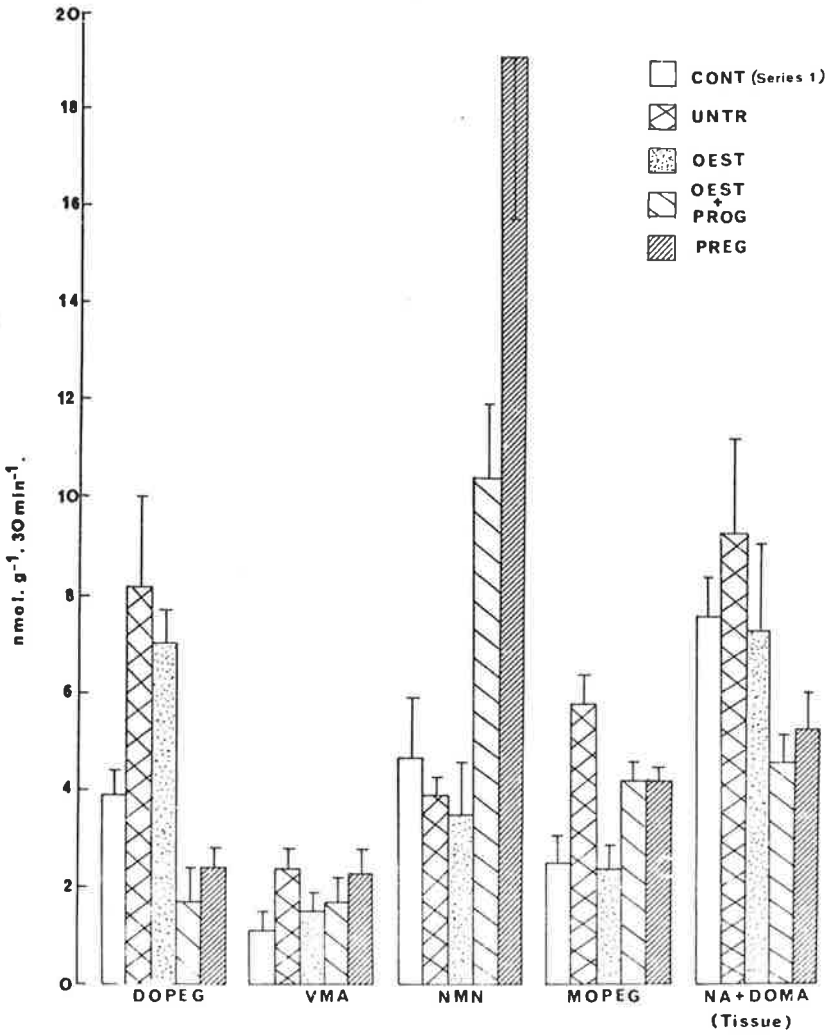

Fig. 7.2 Comparison of the effects of pregnancy and steroid treatment on metabolites of ^{3}H l NA produced by rabbit uterine segments. NA + DOMA represents tissue contents; the other metabolite fractions represent tissue + medium contents. Values are means ± SEs. n = 4 for steroid-treated groups, n = 6 for pregnant rabbits.

DISCUSSION

1. PREGNANT UTERI

From the data presented above it appears that pregnancy greatly enhanced extraneuronal accumulation and metabolism of NA. This follows from the marked predominance of o-methylated metabolites (which were cocaine-insensitive) and the diminished formation of DOPEG per g of tissue. Moreover, there was a minor effect of cocaine on the tissue NA + DOMA fraction and a smaller effect of cocaine (60%) on DOPEG formation in the pregnant uteri than in non-pregnant uteri. From fig. 7.2 it will be seen that there is a striking resemblance between the metabolic pattern produced by pregnancy and that produced by combined progesterone + 17β oestradiol treatment in the oestrogen primed rabbits. Qualitatively both show the same marked increase in NMN formation compared with untreated rabbits, ovariectomized controls and 17β oestradiol treated rabbits. Both show the same decrease in DOPEG formation and tissue NA + DOMA compared with the other three groups. Quantitatively, the effect on NMN is even greater in the pregnant uteri than in those from the combined steroid-treated group. Hence it is likely that the metabolite changes in pregnancy result from the increased level of progesterone in pregnancy leading in turn to a marked increase in the capacity of uterine tissue to o-methylate NA. Although peripheral plasma levels of progesterone reported for pregnant rabbits (Hilliard *et al.*, 1973) are comparable with those achieved with exogenous progesterone in chapter 6, it is not known whether uterine concentrations of progesterone were also comparable in the two groups. Differences in tissue levels might account for the differences in NMN formation between the two groups. Secondly, the increase in size of the uterus is much more marked in the pregnant uteri due to the presence of the developing foetuses and this factor may also account for the greater formation of NMN in the pregnant uteri.

When the data were expressed in terms of NA accumulation and metabolism per whole uterine horn, the effects of pregnancy on o-methylation were even more marked. For example, the total NMN formation in the 30 min period of incubation was 40 fold greater than in the untreated uterus. However, the formation of all metabolites was increased including the cocaine-sensitive component of DOPEG formation. This suggests that there is a small increase in the innervation of the uterus at this stage of pregnancy. However the data in chapter 4 indicated that the total endogenous NA content of the pregnant uterus was not significantly different from that in untreated uteri and Rosengren & Sjoberg (1968) have reported a decrease in NA content of the rabbit uterus at this stage of pregnancy. It is possible that the endogenous NA content may have remained the same but the number of nerves increased if the NA content per nerve terminal was reduced. However in this case it would be anticipated that the cocaine sensitive component of the tissue NA + DOMA fraction would have increased to the same extent as DOPEG formation but this was not so (1.6 fold cf 2.1 fold). Hence the extent of a change in the neuronal component of NA inactivation in the pregnant uterus must be viewed as uncertain at this stage.

The metabolism of ^{3}HlNA by segments from implantation sites and non-implantation sites was qualitatively similar in that DOPEG formation was reduced and NMN formation increased when compared with uteri from non-pregnant, untreated rabbits. However the formation of MOPEG and NMN at implantation sites was less than at non-implantation sites. Factors contributing to these differences may be 1) different local concentrations of the two steroids, oestrogen and progesterone, since additional

secretion is afforded by the foetoplacental unit. and 2) differences in the tissue composition of the two sites. These differences include the specialization of the endometrium and myometrium to form the maternal side of the placenta at the implantation site and the increased vascular supply to this area. Why this should lead to a decrease in o-methylating capacity at this site is not known. Data on the concentrations of MAO and COMT at the two sites is needed as is data on the metabolism of NA in specific structures in the uterus. In particular, the role of the vasculature in NA uptake and metabolism needs to be investigated.

2. COMPARISON WITH OTHER TISSUES

The effect of pregnancy on the metabolism of NA was relatively specific for the uterus since neither ear arteries nor oviducts from the pregnant rabbits showed significant changes in the formation of metabolites or in the tissue accumulation of ^{3}H NA + DOMA compared with non-pregnant rabbits. However pregnancy did alter ovarian uptake and metabolism of NA, both the tissue content of the NA + DOMA fraction and the formation of each of the other metabolites being greatly decreased. Hence this effect was entirely different from that seen in the uterus, and represented a marked decrease in the extraneuronal metabolism of NA since extraneuronal metabolism predominates in the ovary (see chapter 5). Possible factors responsible for this decrease may be a) changes in tissue composition due to corpora lutea formation. Rosengren and Sjöberg (1968) showed a five fold increase in ovarian weight in the pregnant rabbit. If this increase were entirely due to growth of tissue which did not accumulate or metabolize NA then the formation of metabolites per g of tissue would be reduced. b) it is also

possible that endogenous oestrogen and progesterone in the isolated ovaries might have an effect during incubation. Younglai (1975) has demonstrated that the isolated rabbit graafian follicle secretes oestrogen and progestins for at least an hour in the absence of stimulation by luteinizing hormone. Since both agents inhibit extraneuronal uptake of NA in rat heart (Iversen & Salt 1970) it is possible that the endogenous steroids may exert some similar influence on the ovary during incubation. The effect, if it does occur, would be expected to be greater in ovaries from pregnant animals in which oestrogen and progesterone secretion by the ovary is elevated.

CHAPTER 8

DISCUSSION

The aims of this thesis were to investigate whether the metabolism of NA by the rabbit uterus was altered by pregnancy and by the steroid hormones, 17β oestradiol and progesterone, and if so, whether such changes provided any insight into the role of catecholamines in uterine function. The metabolism of NA was investigated in two ways: 1) by measurement of the activity of MAO and COMT in uterine homogenates and 2) by measurement of the metabolites formed from ^{3}H l NA by uterine segments. An index of the degree of sympathetic innervation was obtained by measurement of the endogenous NA concentration of the uterus.

1. SUMMARY OF RESULTS

An initial indication that sympathetic activity in the uterus might alter with pregnancy and ovarian steroid treatment was afforded by the data in chapter 4, in which the endogenous NA concentration of the rabbit uterus was shown to decrease with all treatments except progesterone alone (see table 8.1). A similar indication had already been provided by the data of Falck *et al.* (1969) for the rabbit uterus. As pointed out in the discussion of chapter 4, these changes in NA concentration could be interpreted as a decrease in the density of sympathetic innervation of the uterus as a simple consequence of an increase in organ weight. This interpretation followed from the fact that no treatment altered the endogenous NA content of the whole uterus and the fact that progesterone, alone, did not significantly alter either the weight or the endogenous NA concentration of the rabbit uterus. However this data conflicts with that of Falck *et al.* (1969) who found that the NA content of the whole rabbit uterus was increased

TABLE 8.1 Effect of Treatments on Uterine NA Content

TREATMENTS	NA/g	NA/uterus
Ovariectomized	-	-
17β oestradiol	↓	↔
Progesterone	↔	↔
17β oestradiol + progesterone	↓ ↓	↔
Pregnant	↓↓↓	↔

Footnotes: 1) Effect of treatments are related to ovariectomized controls.

2) ↑ indicates a significant increase,
↓ a significant decrease and
↔ no significant change

TABLE 8.2 Comparison of Effect of Treatments on Enzyme Activities and NA content of the Uterus

TREATMENT	MAO/mg protein	COMT/mg protein	NA/g
Ovariectomized	-	-	-
17β oestradiol	↑ ↑	↑	↓
Progesterone	↑	↔	↔
17β oestradiol + progesterone	↑	↑	↓ ↓
Pregnant	↑	↔	↓↓↓

Footnote: 1) Effects are related to ovariectomized controls

2) ↑ indicates a significant increase,
↓ a significant decrease and
↔ no significant change

by 17β oestradiol and decreased by progesterone or pregnancy. Nevertheless the present data are consistent with those of Miller & Marshall (1965) who could not demonstrate a change in the endogenous NA content of the whole rabbit uterus using a higher dose of 17β oestradiol but for a shorter time. As pointed out in chapter 4, the only apparent difference between the present procedures and those used by Falck *et al.* (1969) was the difference in the strain of rabbit since the dosage regimens were the same and the techniques for measuring NA content were similar.

An indication that the metabolism of NA by the rabbit uterus might be altered by the treatments was gained from the data in chapter 4 on the activities of MAO and COMT in uterine homogenates. 17β oestradiol increased both MAO and COMT activity of the rabbit uterus per mg protein while progesterone increased only the MAO activity. Pregnancy, 28 days after mating, was associated with only a small but significant increase in MAO activity.

The changes in MAO and COMT activity of the uterus bore no simple relationship to the endogenous NA concentration (table 8.2). Thus, although 17β oestradiol treatment increased MAO and COMT activity and decreased NA concentration, progesterone did not alter NA concentration although it did increase MAO activity. Pregnancy greatly decreased NA concentration but caused a slight increase in only MAO activity. Although MAO and COMT activity of the whole uterus was not estimated precisely, 17β oestradiol, 17β oestradiol + progesterone and pregnancy would have increased both MAO and COMT activity of the whole uterus. However, as stated above, these treatments did not decrease the total NA content of the uterus.

Since tyramine was used as a substrate in the estimations of MAO activity, the relative proportions of A and B type isoenzymes of MAO could not be ascertained. Likewise the relative dispositions of either the MAO or COMT activities in nerves or extra-neuronal tissues were not determined. However, the data in chapter 5 on metabolite formation in uterine segments provided a possible indication of the relative dispositions of these two enzymes in the uterus. It was inferred from the relatively large effect of cocaine (29 μmol l^{-1}) on the formation of the deaminated glycol, DOPEG, that this metabolite was formed predominently by MAO within sympathetic nerves. Similarly the lack of effect of cocaine on NMN and O-methylated deaminated metabolite formation was interpreted as evidence both for a largely extraneuronal location of COMT as well as the participation of extraneuronal MAO in the metabolism of NA in this tissue. Evidence for the specificity of cocaine's effect on neuronal uptake of NA at this concentration was presented in the introduction. Evidence for an extraneuronal distribution of COMT and neuronal formation of DOPEG in other tissues was also presented in the introduction. Additional evidence that the reproductive organs conformed to the same general pattern of metabolite formation was shown by the fact that the rabbit oviduct, which was more densely innervated than the uterus, formed relatively more DOPEG and less NMN than the uterus. The rabbit ovary, which is less densely innervated than the uterus, formed relatively more NMN and less DOPEG than the uterus (chapter 5).

The data in chapters 6 and 7 on the metabolite formation by uterine segments from different treatment groups indicated that the metabolism of NA was altered by pregnancy and by pretreatment with 17β oestradiol and progesterone. However, these changes did not appear

TABLE 8.3 Comparison of Effects of Treatments on Metabolite formation and Enzyme Activities in the Uterus

TREATMENT	METABOLITES		ENZYME ACTIVITIES	
	DOPEG/g	NMN/g	MAO/mg protein	COMT/mg protein
Ovariectomized	-	-	-	-
17β oestradiol	a ↑ ↓ b	a ↔ ↔ b	a ↑ b	a ↑ b
Progesterone	N.T. ↓	N.T. ↑	↑	↔
17β oestradiol + progesterone	↓ ↓	↑ ↑	↑	↑
Pregnant	↓ ↓	↑↑ ↑↑	↑	↔

Footnotes: 1) a: comparison is made with ovariectomized controls of series 1.
b: comparison is made with those of series 2.

2) ↑ indicates a significant increase,
↑ a significant decrease and
↔ no significant change.

3) N.T. = not tested

to bear a simple relationship to changes in MAO and COMT activity in the uterus (table 8.3). The most unequivocal finding was that both pregnancy and progesterone treatment were associated with increased NMN formation and this represented a substantial increase in extra-neuronal o-methylation, both on a unit weight basis and total organ basis. However, as indicated above, neither of these procedures affected COMT activity. Moreover 17β oestradiol increased COMT activity but did not affect o-methylated metabolite formation. The lack of an effect on NMN formation in the 17β oestradiol treated group was observed even in cocaine treated preparations in which metabolism is assumed to be almost entirely extraneuronal.

As a result of the above considerations it was concluded in chapters 6 and 7 that the effects of progesterone and pregnancy resulted in increased access of NA to COMT. As in the case of changes in COMT activity in homogenates, changes in NMN formation by the intact cell preparations, did not appear to bear a simple relationship to the endogenous concentration of NA (table 8.4). Thus, 17β oestradiol decreased the endogenous NA concentration of the uterus but did not alter NMN formation while progesterone increased NMN formation without altering endogenous NA concentration. Assuming that the endogenous NA concentration is a measure of the density of sympathetic innervation it was concluded that increased NMN formation due to treatment with progesterone alone was not simply the result of a decrease in the relative amount of neuronal tissue and hence in the proportion of NA metabolized via the neuronal pathway. Diversion of metabolism to the extraneuronal o-methylation pathway might also occur simply as a consequence of stimulation of uterine growth leading to an increase in the amount of extraneuronal tissue. However

TABLE 8.4 Comparison of Effects of Treatments on Metabolite formation and NA content in the Uterus

TREATMENT	per g tissue			per uterus		
	DOPEG	NMN	NA	DOPEG	NMN	NA
Ovariectomized	-	-	-	-	-	-
17β oestradiol	$a_{\uparrow}$ $_{\downarrow}b$	$a_{\leftrightarrow}$ $_{\leftrightarrow}b$	$\downarrow$	$a_{\uparrow}$ $_{\leftrightarrow}b$	$a_{\leftrightarrow}$ $_{\uparrow}b$	$\leftrightarrow$
Progesterone	n.t. $\downarrow$	n.t. $\uparrow$	$\leftrightarrow$	n.t. $\leftrightarrow$	n.t. $\uparrow$	$\leftrightarrow$
17β oestradiol + progesterone	$\downarrow\downarrow$ $\downarrow\downarrow$	$\uparrow$ $\uparrow$	$\downarrow\downarrow$	$\leftrightarrow$ $\leftrightarrow$	$\uparrow\uparrow$ $\uparrow\uparrow$	$\leftrightarrow$
Pregnancy	$\downarrow\downarrow$ $\downarrow\downarrow$	$\uparrow\uparrow$ $\uparrow\uparrow$	$\downarrow\downarrow\downarrow$	$\leftrightarrow$ $\leftrightarrow$	$\uparrow\uparrow\uparrow$ $\uparrow\uparrow\uparrow$	$\leftrightarrow$

Footnotes: 1) a: comparisons are made with series 1 controls,
b: comparisons are made with series 2 controls.

2) $\uparrow$ indicate a significant increase,
$\downarrow$ a significant decrease,
$\leftrightarrow$ no significant change.

3) N.T. = not tested

stimulation of uterine growth of the order achieved with 17β oestradiol alone did not increase NMN formation even in the cocaine treated preparations. Nevertheless the larger increase in extra-neuronal tissue relative to nerves seen with the combined steroid treatment and in pregnancy may have accounted for the finding that these procedures yielded the highest rates of NMN formation.

In contrast to NMN, the formation of DOPEG per g of tissue, appeared to be more simply related to the endogenous NA concentration of the uterus as shown by the positive correlation between these two variables (table 8.4) i.e. treatments which increased uterine weight tended to decrease the amount of DOPEG formed per g of tissue but not per whole uterus. This was explained in terms of the decrease in density of sympathetic innervation resulting in a decrease in the amount of neuronal MAO available for DOPEG formation. A possible exception was 17β oestradiol treatment in one series of experiments in which there was a significant increase in the formation of DOPEG per g of tissue and per whole uterus. Unfortunately, the concentration of NA was not measured in this series of experiments.

However, as with o-methylated metabolite formation, changes in DOPEG formation did not bear an obvious relationship to changes in enzyme activity. Although, in one case, 17β oestradiol treatment increased both DOPEG formation and MAO activity, progesterone alone increased MAO activity but not DOPEG formation and small increases in MAO activity in the combined steroid treatments and pregnancy were associated with decreased formation of DOPEG per g of uterus. However, as indicated already neuronal MAO activity was not measured separately

and it is not known whether it would show a relationship with DOPEG formation.

Limited comparisons with other reproductive tissues indicated that neither the rabbit oviduct nor ovary showed the same types of change in metabolite formation as seen in the uterus. The metabolism of NA in the rabbit oviduct was not altered by pregnancy. In the ovary the pattern of metabolite formation was unchanged but in contrast to the uterus the rate of metabolism was markedly reduced.

Neither of the steroid treatments nor pegnancy altered the metabolism of NA by the rabbit ear artery. As discussed in chapter 6, the specificities of these treatments on uterine metabolism may reflect the fact that non-target tissues such as the ear artery neither may achieve the same tissue concentrations of steroid nor undergo the same changes in organ weight as the uterus. Although the oviduct is a target tissue for these steroids, its metabolism of NA appears to be predominantly neuronal and its density of innervation has been reported to remain constant during pregnancy (Rosengren & Sjoberg 1968).

2. COMPARISON WITH OTHER SPECIES

The present results confirm other reports that the rabbit uterus contains predominantly NA (Sjöberg 1967, Miller & Marshall 1965) like the guinea pig (Falck *et al.*, 1974) but unlike the rat uterus which has a relatively high A content which increases during oestrous and pregnancy (Wurtman *et al.*, 1963, Spratto & Miller 1968).

Although in the present study no effect of pregnancy or steroids on total uterine NA content was observed, changes in NA concentration being due to effects on organ weight, there are a number of reports of changes in NA content of the whole uterus under these conditions in both the rabbit and other species such as guinea pig and humans (Falck *et al.*, 1974, Nakanshi *et al.*, 1968). As discussed previously, differences in the strain of rabbit used combined with the stage of pregnancy sampled are the only explanations which can be offered to account for these differences. Bell (1974) was also unable to demonstrate a change in NA content of the guinea pig uterus at a stage of pregnancy when he observed loss of perivascular catecholamine fluorescence. It may be helpful to measure specific areas of the uterus rather than the total organ to increase the sensitivity of the measurement of tissue catecholamines, particularly during pregnancy when the concentration of NA is low. Hanberg, Pennefather & Farrar (1978) measured the NA content of the longitudinal muscle of the guinea pig uterus and although unable to detect changes in a pooled sample of animals during the oestrous cycle, they demonstrated a decrease in NA content associated with increased progesterone levels if animals of similar body weight were used.

Although the effects of steroids and pregnancy on NA content of the whole rabbit uterus differed from those reported previously (Sjöberg 1967, Falck *et al.* 1969) the changes in NA concentration of the uterus under these conditions were similar.

The rabbit uterus was similar to the rat in that MAO activity greatly exceeded COMT activity (Wurtman *et al.*, 1964). However the rabbit uterus differed from the rat and human uterus in that MAO activity was increased by 17β oestradiol treatment in the rabbit rather than decreased. However the response to progesterone treatment was similar in all three species, MAO activity increasing (Southgate *et al.*, 1969, Holzbauer & Youdim 1973). The different response to 17β oestradiol is unlikely to be due to differences in the substrates used and to differences in the preparation of the enzyme since Southgate *et al.* (1969) demonstrated the effect of 17β oestradiol on MAO activity in mitochondrial preparations as well as in straight homogenates and showed similar changes using substrates for both A and B type isoenzymes, including tyramine (Southgate 1972). As discussed in chapter 4, a competitive inhibitory influence of 17β oestradiol on uterine MAO activity as reported by Collins & Southgate (1970) for the rat uterus would probably not have been detected in the present study since preparatic of the enzyme would presumably have removed much of the 17β oestradiol present in the tissue.

The rabbit also differed from the rat in that uterine COMT activity did not increase during pregnancy as it did in the rat (Wurtman *et al.*, 1964). The effects of steroids on uterine COMT activity in the two species are difficult to compare since data is available only for the effects of the oestrous cycle on rat uterine COMT. No data is available on human uterine COMT activity for comparison with the rabbit.

Comparative data for other species on metabolite formation in uterine segments is limited. Green & Miller (1966a) demonstrated that, as in the rabbit uterus, the accumulation of ^{3}H NA and ^{3}H A per g of uterus in the rat, declined with increasing uterine weight but the accumulation of amine per whole uterus did not change with the uterine weight.

A comparison of their results with these in the rabbit is difficult since they were unable to demonstrate metabolites of NA in any of their uterine slices. O-methylated metabolites, but not deaminated metabolites, of A were detected and their formation shown to be greater during oestrous than dioestrous. The decreased formation of o-methylated metabolites at dioestrous in the rat, when progesterone levels are elevated (Holzbauer & Youdim 1973) appears to differ from the increase in o-methylation in the rabbit uterus after progesterone treatment. However more specific comparisons of the two species with respect to the effect of progesterone need to be made. Green & Miller (1966a) used ^{3}H dl NA and ^{3}H dl A at low substrate concentrations (30 nmol l^{-1}) compared with the ^{3}H l NA (1.2 µmol l^{-1}) used in the present study. A factor accounting for their inability to detect deaminated catechols may have been the low density of innervation in the rat uterus compared with the rabbit (0.92 nmol g^{-1}; Falck *et al.*, 1974 cf. 2.88 nmol g^{-1} endogenous NA respectively) combined with their low substrate concentration. The lack of deaminated metabolites of A may not be surprising since A is predominantly o-methylated in other tissues such as dog saphenous vein (Brandão *et al.*, 1978). Thus there is a need to re-examine the inactivation of NA by rat uterine segments

using the more sensitive separative techniques which have become available during the past ten years.

In summary, the limited interspecies comparisons available indicate that the rabbit uterus differs from that of the rat and human in the response of its NA metabolizing enzymes to ovarian steroids and pregnancy. However since the number of species studied is small and the number of studies of metabolite formation in whole cell preparations even smaller, the significance of these differences cannot be assessed at this stage.

3. FUNCTIONAL SIGNIFICANCE

The possible functional significance of the sympathetic innervation on female reproductive function has been briefly outlined in the introduction. The present findings on NA inactivation by the rabbit uterus do not offer any critical information on the various hypotheses concerning uterine innervation and a possible modulating role in gamete transport and maintenance of pregnancy. The functional significance will be considered only in so far as it points to further ways in which the intensity of the effects of sympathetic stimulation and circulating catecholamines on the uterus may be modified in addition to the two major mechanisms of a) changes in density of innervation and b) changes in the α and β receptor activity of the uterus.

The increased extraneuronal inactivation of NA in the rabbit uterus occurred in treatments in which endogenous NA concentration decreased and β receptor activity has been reported to increase. Thus pregnancy and 17β oestradiol plus progesterone treatment led to decreased density of sympathetic innervation and increased extraneuronal inactivation.

Coupled with a decrease in myogenic conduction in uterine smooth muscle resulting from the effects of progesterone (Csapo 1969) one might anticipate a decrease in the influence of the sympathetic nerves on uterine motility as pregnancy advances. Such an hypothesis is contrary to the suggestion that the sympathetic nerves provide a tonic inhibitory influence in the uterus at least in mid- to late-pregnancy when the density of innervation is greatly decreased. The role of the innervation to the uterus may be more important during conception and early pregnancy before the density of innervation declines markedly. It has been suggested that the change to β-mediated relaxation in response to catecholamines in the pregnant uterus may be a protective mechanism against increases in circulating catecholamines (Pauerstein & Zaunder 1970). The increased extraneuronal o-methylation of NA by the pregnant uterus may well be part of such a protective mechanism. Although Stone *et al.* (1960) reported changes in plasma catecholamines in the second trimester of pregnancy the three fold increase in A observed was matched by a three fold decrease in NA concentration and the NA and A concentration returned to nonpregnant levels in the third trimester. Similar increases in plasma A and decreases in plasma NA concentrations have been reported for the rat by Green & Miller (1966c). However changes in uterine responsiveness may be more important in situations in which plasma catecholamines fluctuate more rapidly. Stressful situations are likely to result in sudden increases in circulating catecholamines against which the uterus may need to be protected. Increases in extraneuronal o-methylation may also be more important in the inactivation of circulating A since A has been shown to be o-methylated to a greater extent than NA in the tissues where such a comparison has been made (dog saphenous vein, Brandão *et al.*, 1978).

Certain exceptions to such an hypothesis would need explanation in that 1) the cat uterus develops α mediated contractile responses to catecholamines during pregnancy and 2) there is some evidence from Green & Miller's work (1966a) that progesterone dominance may decrease o-methylation in the rat uterus although this needs further verification.

Bell (1972) has also suggested that the decrease in density of sympathetic vasomotor nerves in the guinea pig uterus is a protective mechanism against stress-induced ischaemia of the foetus and placenta during pregnancy. However, circulating catecholamines released during stress presumably would constrict uterine blood vessels since these have a preponderance of α receptors and do not show an increase in β receptor activity during pregnancy as does the myometrium. There is increasing evidence that extraneuronal inactivation is important in vascular sensitivity to catecholamines (de la Lande, 1975). Although it has not been studied separately, it is possible that extraneuronal inactivation may also be important in uterine vascular sensitivity to catecholamines, particularly in the pregnant animal.

4. FURTHER EXPERIMENTS

(i) Since MAO activity was measured using tyramine as a substrate it was difficult to extrapolate the effects of the steroids on enzyme activity to the formation of deaminated metabolites in uterine segments. There is a need to examine the effect of steroid treatment and pregnancy on MAO activity as estimated using different substrates, particularly NA. In view of the difficulty experienced using NA as a substrate in homogenates (Appendix 2), it may be profitable to examine

the effect of specific inhibitors such as clorgylene and deprenil on the formation of deaminated metabolites in uterine segments *in vitro* in order to distinguish the effects of different isoenzymes of MAO.

(ii) Further characterization of the effect of the steroids and pregnancy on inactivation of NA by the uterus might be achieved by examining the kinetics of uptake into and efflux from different compartments in uterine tissue.

(iii) In these experiments, no evidence of an inhibitory effect of the steroids on extraneuronal uptake of NA was obtained in uteri from animals pretreated with the steroids. However, the effect of these same exogenous steroids on extraneuronal uptake *in vitro* should be studied to see whether any of their *in vivo* effects could be related to their effects on extraneuronal uptake. Since ISO is mainly accumulated extraneuronally (Hertting 1964) it would be useful as a substrate in any such study.

(iv) The changes in metabolism of NA in the uterus resulting from steroid treatment need to be localized. Since the uterus was not subdivided, the relative roles of the endometrium, myometrium and blood vessels were not characterized in the rabbit. In the human, the endometrium has been shown to possess MAO activity which alters with the stage of the sexual cycle (Southgate *et al.*, 1969). The role of the endometrium could be studied by separating it mechanically from the rest of the uterus. Pennefather (1978) has separated the longitudinal muscle of the guinea pig myometrium from the rest of the uterus and suggested that the costo-uterine muscle, which is continuous with this layer of the myometrium, might be used as a model for it.

The role of the blood vessels in uterine inactivation of catecholamines is more difficult to determine. The problem might be approached by studies of the inactivation of NA in perfused uterine preparations. It is possible that the vascular contribution to neuronal inactivation of NA may be assessed by surgical denervation since, unlike the nerves supplying the myometrial muscle, the blood vessels are supplied by long post-ganglianic fibres in the rabbit (Sjöberg, 1967).

(v) A comparison of the rabbit, which is an induced or reflex ovulator, with other spontaneously ovulating species such as the rat and guinea pig is necessary, particularly in suggesting a function for changes in uterine metabolism. Moreover, such a comparative study would be useful in that there is species variability in the density and origin of the uterine innervation. In the rat, since the sympathetic nerves are confined mainly to the vasculature, information may be gained on the role of the blood vessels in neuronal inactivation of NA in the uterus when compared with other species. In the guinea pig Issac *et al.* (1969) and Thorbert *et al.* (1977) have demonstrated that the ovarian end of the uterus is innervated by long postganglianic fibres from the ovarian nerves. Surgical denervation of this area of the uterus should be possible in the guinea pig and may provide further information on the relative roles of the nerves and extraneuronal tissue in metabolism of NA in the uterus.

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APPENDIX 1

Drugs	Manufacturer
l-adrenaline bitartrate	Koch Light Laboratories
cocaine hydrochloride	MacFarlane-Smith
clorgylene	May and Baker
3,4 dihydroxyphenyl glycol	Sigma
3,4 dihydroxymanelic acid	Sigma
Guanethidine sulphate	
6 hydroxydopamine hydrobromide	Sigma
dl isoprenaline hydrochloride	Sigma
dl-7-^{3}H isoprenaline hydrochloride (specific activity 5-15 Ci $(mmol)^{-1}$)	Amersham
3,methoxy, 4,hydroxy phenyl glycol, piperazine salt	Sigma
nialamide	Pfizer
l-noradrenaline bitartrate	Koch Light Laboratories
l-7-^{3}H noradrenaline, (specific activity 5-15 Ci $(mmol)^{-1}$)	Amersham
dl normetanephrine hydrochloride	Sigma
17β oestradiol (oestra-1,3,5(10) - triene-3, 17β diol)	Steraloids
pentobarbitol (Sagatal)	May and Baker
progesterone (Δ^{4}-pregnene-3, 20-dione)	Steraloids
S-adenosyl-l-methionine	Amersham
S-adenosyl-l-^{14}C methyl methionine (specific activity 56 mCi $(mmol)^{-1}$)	Amersham
Tyramine hydrochloride	Sigma

Drugs	Manufacturer
^{3}H Tyramine hydrochloride (specific activity 6.8 Ci $(mmol)^{-1}$)	Amersham
UO521 (3', 4'-dihydroxy-2-methyl propiophenone)	Upjohn
vanillyl mandelic acid	Sigma

All other reagents were analytical grade purity.

APPENDIX 2

Monoamine Oxidase Assays in Rabbit Uterus

Since the assay employed in chapter 4, used ^{3}H tyramine as a substrate for MAO, no indication was obtained of the relative amounts of different isoenzymes of MAO. In particular, MAO type A, described by Johnston (1968) which has a high affinity for NA, was not estimated separately. In this appendix, some preliminary results are presented from two attempts to estimate type A MAO in the rabbit uterus. In one case, the method of Jain *et al.* (1973) employing NA as a substrate was used; in the second case, the inhibitor clorgylene was used in conjunction with tyramine as a substrate.

1) Use of ^{3}H dl NA as a substrate

The method was that described by Jain *et al.* (1973) with the exception that ^{3}H dl NA was used instead of ^{14}C NA. The reaction mixture consisted of 30 µl tissue homogenate, 20 µl ascorbic acid (2.9 mol l^{-1}) 20 µl phosphate buffer (pH 7.4, 0.1 mol l^{-1}) and 40 µl of ^{3}H dl NA (final concentration 0.5 mmol l^{-1}; specific activity 2.7 µCi (µmol^{-1}) and 90 µl water. The reaction was carried out at 36^{o}C in O_2 filled glass tubes, with continuous shaking. After 15 mins the reaction was stopped by the addition of 0.10 ml HCl/ml (i.e. 20 µl). Neutral and acidic metabolites were separated using Dowex 50 x-8 (H), 200-400 mesh, of dimensions 0.8 x 3 cms. The first 5 ml of effluent was collected and a 0.5 ml aliquot counted for radioactivity. Table 1 compares the radioactivity present in the Dowex effluent obtained after incubation of liver homogenates and

uterine homogenates. The blank was obtained by stopping the reaction at zero time with HCl.

Table 1

	DPM (500 µl)$^{-1}$	nmol product (mg protein)$^{-1}$hr^{-1}
Liver	1.39 ± 0.20 x 10^4	13.2 ± 1.8
Uterus	420 ± 53	-
Blank	390 ± 41	-

Since values for the uterus were not distinguishable from the blank, a second method for estimating type A, MAO in the uterus was attempted.

2) Use of Clorgylene to Estimate A/B type MAO

Type A and B MAO have been identified in rat cerebral hemispheres by Goridis and Neff (1971) by the use of clorgylene which has a higher affinity for inhibiting type A MAO. They obtained a plateau region in the inhibition curve produced by clorgylene at 10^{-6} to 10^{-7} mol l^{-1} clorgylene, corresponding to 55% inhibition; 100% inhibition was produced only at 10^{-3} mol l^{-1} clorgylene. Their estimate was 55% type A and 45% type B MAO in rat cerebral hemispheres.

A similar method was applied to the rabbit uterus with the exception that ^{3}H tyramine was used as a substrate instead of ^{14}C tyramine, and the products were extracted by the method of Jarrott (1971).

Method:

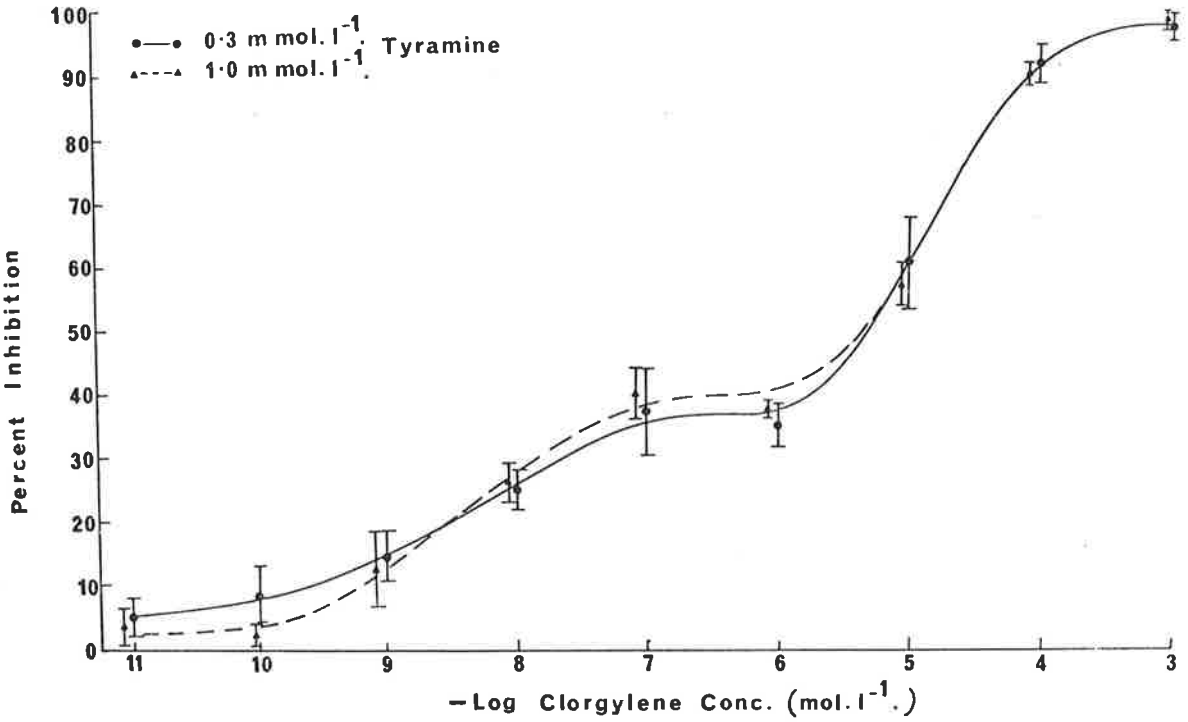
i) Tissue preparation

Rat cerebral hemispheres and rabbit uteri were homogenized in 0.25 mol l^{-1} sucrose (1/20 weight/volume) using a glass homogenizer with a teflon pestle. The homogenate was centrifuged at 700 g for 20 mins and the supernatant was then centrifuged at 11,000 g for 20 min. The pellet was washed once with 0.67 mol l^{-1} Na/K phosphate buffer, pH 7.2 and then resuspended in the same buffer (1/20 weight/volume).

ii) Assay conditions

(a) The purity of the substrate, ^{3}H tyramine, was checked by descending paper chromatography. A single spot was obtained with Ninhydrin which corresponded with unlabelled tyramine and ^{14}C tyramine (Amersham). A single peak of radioactivity was obtained which corresponded to the peak for ^{14}C tyramine. The ^{3}H tyramine was then diluted with unlabelled tyramine in Na/K phosphate buffer (0.2 mol l^{-1}, pH 7.8) to a final concentration of 2 mmol l^{-1} (7.6×10^{6} DPM ml^{-1}).

(b) The mitochondria were preincubated with clorgylene 10^{-12} to 10^{-3} mol l^{-1} for 15 min at 20°C (25 µl tissue and 25 µl clorgylene) 50 µl of ^{3}H tyramine was then added to give a final concentration of 1 mmol l^{-1} (3.8×10^{5} DPM). The tubes were gased with O_2 and capped. Tubes were incubated with shaking at 37°C for 15 min. The reaction was stopped with 10 µl of HCl (3 mol l^{-1}) on ice.



Appendix 2 fig. 1 Inhibition of MAO activity by different concentrations of clorgylene in homogenates from rat cerebral hemispheres. The plateau at 10^{-6} to 10^{-7} mol l^{-1} clorgylene indicates maximal inhibition of type A MAO.

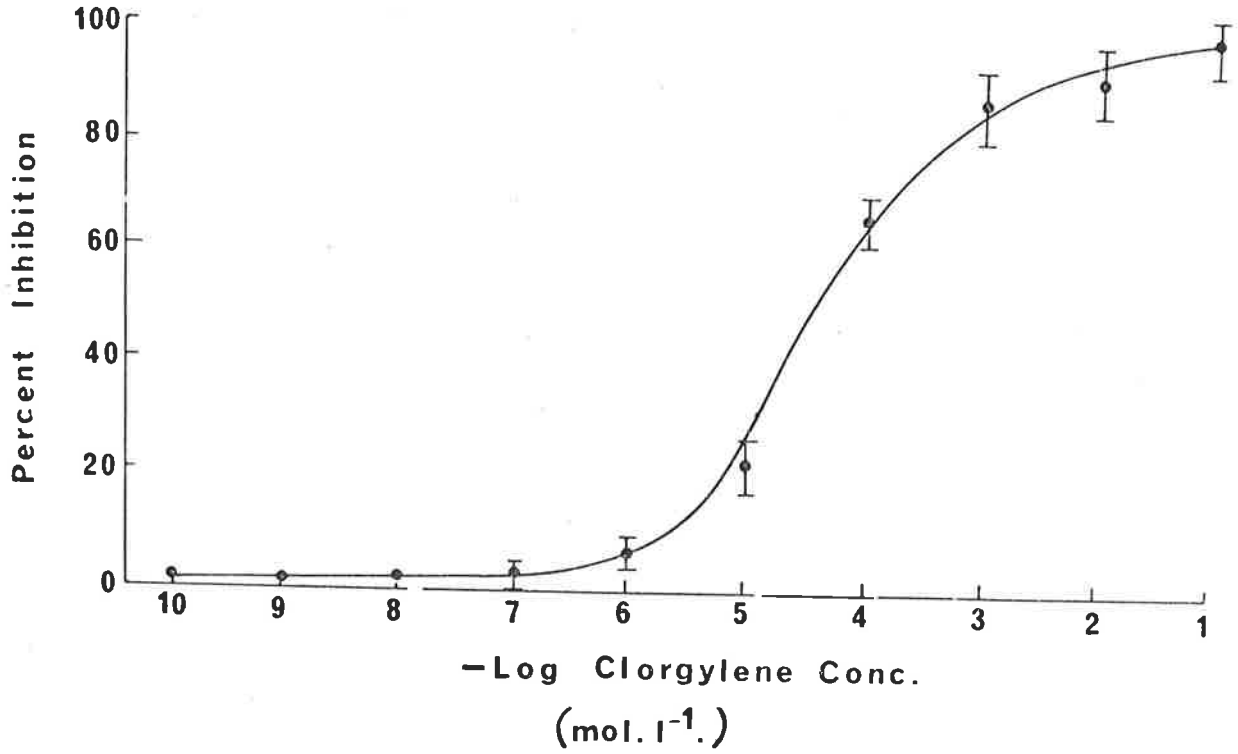
(c) Extraction of Products. 500 µl of ethyl acetate was added to each tube and shaken for 5 min. The two phases were separated by centrifugating for 5 min. 400 µl of the ethyl acetate phase was added to 100 µl of 0.3 mol l^{-1} HCl. After shaking and centrifuging, 300 µl of the ethyl acetate phase was added to 15 mls of scintillation fluid and counted.

Results

Fig. 1 shows the results obtained from rat cerebral hemispheres. It will be seen that a plateau occurred at 10^{-6} to 10^{-7} mol l^{-1} clorgylene as observed by Goridis and Neff (1971). However this result indicated that only 35% of the total MAO activity could be attributed to the type A isoenzyme, a value substantially less than that obtained by Goridis and Neff. The results were similar for both 0.3 mmol l^{-1} and 1 mmol l^{-1} tyramine. In fig. 2, the results obtained from rabbit uteri indicated that very little type A MAO was present.

Discussion

The results presented here indicate that the rabbit uterus may contain very little type A, MAO. However since the present estimate of type A MAO for rat cerebral hemispheres is substantially lower (20%) than that estimated by Goridis and Neff (1971) some caution should be exercised in reaching a conclusion as to the amount of type A MAO present in the rabbit uterus. Obviously it is much less than that in rat cerebral hemispheres. A more sensitive assay using a type A substrate, or the use of clorgylene in isolated uteri incubated with radiolabelled NA, may provide more conclusive results.



Appendix 2 fig. 2 Inhibition of MAO activity by different concentrations of clorgylene in homogenates of rabbit uterus.

APPENDIX 3

Chemical Sympathectomy

Since surgical denervation of the uterus is exceedingly difficult in the rabbit due to the ganglia being situated on the wall of the uterus and vagina (Marshall, 1970) attempts were made to denervate the rabbit uterus by using chemical denervating agents. 6-hydroxydopamine has been reported to denervate the rabbit uterus (Hervonen & Kanerva 1974). Guanethidine has been reported to produce a long lasting sympathetic denervation of reproductive tissues in the rat (Burnstock *et al.*, 1971). It was intended that these agents would provide additional evidence on the neuronal and extraneuronal origins of metabolites of ^{3}H l NA in the uterus. Preliminary results of these treatments are presented below.

Methods:

(a) 6-hydroxydopamine

The method of Finch *et al.* (1973) was used. Rabbits were injected intravenously with 6-hydroxydopamine (50 mg/Kg) twice on day 1, into the marginal ear vein. 6-hydroxydopamine was dissolved just prior to use. On day 8, rabbits were injected twice with 100 mg/Kg at an interval of approximately 8 hours. They were sampled on day 9.

(b) Five rabbits received guanethidine (30 mg/Kg) intraperitoneally, daily for 6 weeks according to the method of Burnstock *et al.* (1971). Two of the rabbits received an extra week of injections of 60 mg/Kg daily, since sampling of the first three rabbits indicated that their

uteri were not denervated. Control rabbits received the vehicle (saline) for the same period of treatment.

After both of these treatment regimes, uteri and ear arteries were assayed for endogenous NA content as described in chapter 2. Ear arteries and segments of uteri were incubated with ^{3}H l NA (1.2 μmol l^{-1}) for 30 min at 37^{o}C and the metabolites formed were separated by TLC.

Results:

(a) 6-Hydroxydopamine

Treatment proved unsatisfactory, at least using this treatment regime. There was a high mortality rate, only 50% of animals surviving to day 9. The cause of death was not ascertained but death was rapid, occurring within minutes of injection. Those animals which survived to day 9 did not appear to be denervated as indicated by the data in table 1. Endogenous NA contents of uteri and ear arteries from rabbits treated with 6-hydroxydopamine were not significantly different from those in control animals.

(b) Guanethidine

The data in table 2 indicate that guanethidine treatment significantly reduced the endogenous NA content of the rabbit ear artery but not of the uterus. Although the NA concentration in ear arteries was reduced, complete denervation was not achieved as evidenced by the remaining 4.22 ± 0.9 nmol g^{-1} NA. It should be noted that the value for control arteries was approximately half that reported by Head (1976).

In spite of a reduction in endogenous NA content of ear arteries, the data in table 3 indicate that the guanethidine treatment had no effect on the accumulation and metabolism of ^{3}H l NA in ear arteries. The ^{3}H l NA accumulated and DOPEG formed was less in uteri from the guanethidine-treated rabbits than in controls but the low number did not allow any definite conclusions to be drawn.

Comments:

It was surprising that the 6-hydroxydopamine treatment did not denervate rabbit uteri since Hervonen & Kanerva (1974) reported denervation of the rabbit uterus after a single intravenous injection of 50 mg/Kg 6-hydroxydopamine. Although the guanethidine treatment used in these preliminary experiments did not denervate uteri or ear arteries, it is possible that a higher dose of guanethidine may produce the desired sympathectomy since some effect was obtained on ear arteries. It is possible that the rabbit may need a different dosage regime from that used by Burnstock *et al.* (1971) for the rat.

APPENDIX 4

Composition of Krebs Bicarbonate Solution

NaCl	120	mmol l^{-1}
KCl	4.7	mmol l^{-1}
Na_2CO_3	25	mmol l^{-1}
glucose	5.5	mmol l^{-1}
KH_2PO_4	1.0	mmol l^{-1}
$CaCl_2$	2.5	mmol l^{-1}
MgCl	1.1	mmol l^{-1}
EDTA	10.8	µmol l^{-1}
ascorbic acid	290	µmol l^{-1}

TABLE 1. Effect of 6-hydroxydopamine in NA content of ear arteries and uteri.

Treatment	Endogenous NA content	
	Ear Arteries (nmol g^{-1})	Uteri (nmol $uterus^{-1}$)
Control	10.30 ± 0.60	5.10 ± 0.50
6-OH-Dopamine	9.61 ± 0.72	4.68 ± 0.70

Footnotes: (1) NA content was assayed by automated fluorescent assay described in chapter 2.

(2) Values are means ± SEs for n = 4.

(3) nmol $uterus^{-1}$ refers to 2 uterine horns.

TABLE 2. Effect of Guanethidine on NA content of Ear Arteries and uteri.

Treatment		Endogenous NA content	
		Ear Arteries (nmol g^{-1})	Uteri (nmol $uterus^{-1}$)
Control	1	6.35	4.00
	2	8.76	3.55
	3	9.24	4.66
	4	9.65	5.59
		8.50 ± 0.74	4.45 ± 0.44
Guanethidine	1	4.06	3.80
(a) 6 weeks	2	4.76	3.65
(30 mg/Kg)	3	3.00	5.00
(b) +1 week	1	5.35	4.90
(60 mg/Kg)	2	3.94	4.88
		4.22 ± 0.40	4.45 ± 0.30

TABLE 3. Effect of Guamethidine on metabolism of ^{3}H l NA in rabbit ear arteries and uteri.

Treatment		Metabolites				
		NA + DOMA	DOPEG	VMA	NMN	MOPEG
EAR ARTERIES						
Control (n = 4)	Tiss	5.33±0.47	0.14±0.02	0.03±0.01	0.08±0.02	0.05±0.003
	Med.	-	1.16±0.25	0.12±0.08	0.26±0.06	0.26±0.02
Guaneth						
a (n = 3)	Tiss	7.17±0.13	0.16±0.03	0	0.04±0.03	0.06±0.01
	Med	-	1.48±0.09	0.17±0.04	0.12±0.05	0.30±0.02
b	Tiss1	5.03	0.23	0.01	0.13	0.08
	2	6.36	0.32	0.03	0.12	0.09
	Med 1	-	2.11	0.28	0.31	0.38
	2	-	3.65	0.51	0.45	0.47
UTERI						
Control (n = 4)	Tiss	14.4±3.04	0.81±0.12	0.60±0.12	0.90±0.26	1.63±0.77
	Med	-	5.49±0.58	1.20±0.22	1.34±0.79	2.41±0.53
Guaneth						
a (n = 3)	Tiss	4.9±0.6	0.41±0.09	0.53±0.10	1.45±0.23	1.03±0.13
	Med	-	3.71±0.52	0.81±0.21	1.81±0.58	2.04±0.43
b	Tiss1	7.01	0.45	0.73	2.89	1.33
	2	5.41	0.36	0.69	2.01	0.85
	Med 1	-	2.34	1.55	3.48	3.22
	2	-	1.80	2.09	2.77	2.41

Footnotes: (1) Values are expressed as nmol g^{-1} $(30\ min)^{-1}$. Rabbit ear arteries are related to wet weight, uteri to dry weight.

(2) a = 30 mg Kg^{-1} guanethidine for 6 weeks

b = 30 mg Kg^{-1} guanethidine for 6 weeks + 60 mg Kg^{-1} for 1 week.