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(Signed).....C.R. Triggle.......

PERMANENT ADDRESS:

C.O. DEPT. BIOCHEMICAL PHARMACOLOGY,
S.U.N.Y.A.B.
BUFFALO
NEW YORK STATE 14214

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THE MODE OF ACTION OF COCAINE IN PRODUCING SUPERSENSITIVITY TO NORADRENALINE IN THE HUMAN UMBILICAL ARTERY

by

CHRISTOPHER ROBERT TRIGGLE

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

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THE UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled THE MODE OF ACTION OF COCAINE IN PRODUCING SUPERSENSITIVITY TO NORADRENALINE IN THE HUMAN UMBILICAL ARTERY submitted by CHRISTOPHER ROBERT TRIGGLE in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

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Supervisor

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....

....
External Examiner

Date April 8/72

ABSTRACT

Fluorescent histochemical studies have been used to show that the extra-abdominal portion of the human umbilical cord is devoid of adrenergic nerves. The use of both fluorescent and biochemical assay techniques have indicated that extraneuronal uptake of noradrenaline in the human umbilical artery is considerably less sensitive to inhibition by normetanephrine, phenoxybenzamine and temperature changes than the extraneuronal uptake which is reported to occur in innervated tissues. Cocaine, except at high concentrations, was also found to have no effect on extraneuronal noradrenaline uptake; similar findings have been reported by others using innervated tissues.

Since cocaine has no effect on noradrenaline uptake in the human umbilical artery, this provides a useful preparation with which to investigate the mode of action of cocaine in producing noradrenaline supersensitivity. It was found that cocaine could potentiate the responses to noradrenaline and, to a lesser degree, tyramine and 5-hydroxytryptamine, in helically-cut umbilical artery strips. The responses to potassium and histamine were not significantly affected by cocaine. By the use of fairly selective antagonists, phentolamine and 2'[3 dimethyl amino propyl thio]cinnamonilide (DPTC), it was found that there is little indication of an interaction between noradrenaline and the 5-hydroxytryptamine receptor, or 5-hydroxytryptamine and the α-receptor. These results suggest that, in this tissue, cocaine can increase the responses to noradrenaline, tyramine and 5-hydroxytryptamine

by a postsynaptic mechanism which appears to be selective towards the noradrenaline-mediated response. This selective increase in the response mediated by noradrenaline may be explained by an allosteric alteration of the α-adrenergic receptor system; possibly a non-selective increase in smooth muscle sensitivity is also involved.

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TABLE OF CONTENTS

	Page
I. INTRODUCTION	1
A. GENERAL INTRODUCTION	2
B. STRUCTURE AND FUNCTION OF THE PLACENTA AND UMBILICAL CORD	2
C. POSSIBLE MECHANISMS OF POST-PARTUM CHANGES	3
D. INNERVATION OF THE UMBILICAL CORD AND PLACENTA	7
E. PHARMACOLOGY OF PLACENTA AND UMBILICAL CORD	11
F. POSSIBLE MECHANISMS OF ACTION OF COCAINE	15
1. Inhibition of inactivation of catecholamines by cocaine	15
a. Blockade of MAO and COMT	15
b. Inhibition of catecholamine uptake	16
2. Direct action on the effector cell	17
G. EXTRANEURONAL UPTAKE OF NORADRENALINE	23
H. ALLOSTERISM AND DRUG RECEPTORS	29
1. Allosteric changes and cocaine action	29
2. General theories of allosterism, conformational changes, and cooperativity	29
3. Allosteric changes and the cholinergic receptor	32
4. Allosteric changes and the adrenergic receptor	33
I. EFFECT OF COCAINE ON SMOOTH MUSCLE CALCIUM	34
J. CALCIUM AND THE α-RECEPTOR	36
K. COCAINE AND THE α-RECEPTOR	37
L. AIMS OF THE RESEARCH	39

Page

II. METHODS 42

A. PERFUSION OF WHOLE PLACENTA 43

B. PREPARATION OF ISOLATED UMBILICAL ARTERY STRIPS 45

C. CATECHOLAMINE ANALYSIS 48

1. Reagents 49

a. Aluminum oxide 49

b. 0.4M perchloric acid 51

c. 0.2M acetic acid 51

d. 0.5M Tris buffer, pH 9.0 51

e. Acetate buffer, pH 7.0 51

f. Iodine 51

g. Alkaline sulphite 51

h. Distilled water 51

2. Preparation of samples 52

3. Formation of the fluorescent trihydroxyindole derivatives 52

a. Procedure 52

b. Calculation of noradrenaline 54

c. Differential measurement of adrenaline and noradrenaline 54

d. Determination of percentage recovery 56

e. Effect of normetanephrine, metaraminol, cocaine, phenoxybenzamine, ouabain and parnate on the trihydroxyindole procedure 56

D. NORADRENALINE UPTAKE EXPERIMENTS 57

E. FLUORESCENCE MICROSCOPY 57

F. STATISTICS 63

Page

III. RESULTS 64

A. WHOLE ORGAN PERFUSION EXPERIMENTS 65

1. Responses to tyramine 65

2. Effect of cocaine 68

B. NORADRENALINE ANALYSIS OF PLACENTA AND UMBILICAL CORD 68

C. FLUORESCENCE MICROSCOPY OF UMBILICAL CORD TISSUE 73

1. Normal tissues 73

2. Incubated tissues 76

3. Effect of MAO inhibition on noradrenaline uptake 84

4. 5-hydroxytryptamine accumulation in the umbilical cord 84

5. Distribution of noradrenaline in the placental villi 87

6. Effect of temperature on noradrenaline inhibition in the umbilical cord 87

7. Effect of phenoxybenzamine on noradrenaline distribution in the umbilical cord 87

8. Effect of cocaine on noradrenaline distribution in the umbilical cord 88

9. Effect of normetanephrine on noradrenaline distribution in the umbilical cord 88

10. Loss of accumulated noradrenaline from the tissue 88

D. UPTAKE OF NORADRENALINE BY ISOLATED UMBILICAL ARTERIES 89

1. Accumulation of noradrenaline 89

2. Loss of noradrenaline from tissue 89

3. Effect of temperature on the uptake of noradrenaline 92

4. Effect of cocaine on uptake of noradrenaline 92

5. Effect of various agents on uptake of noradrenaline 92

a. Phenoxybenzamine 95

Page

b. Ouabain pretreatment 95

c. Normetanephrine 95

d. Metaraminol 95

e. Parnate (MAO inhibitor) pretreatment 95

E. ISOLATED TISSUE EXPERIMENTS 95

1. General Pharmacology 95

2. Effect of cocaine on agonist response 101

3. Effect of phentolamine on responses to agonists 112

4. Effect of propranolol on responses to agonists 112

5. Effect of noradrenaline on induced tyramine responses 118

6. Effect of DPTC on responses to 5-hydroxytryptamine, noradrenaline and tyramine 118

7. Cocaine protection against receptor blockade by phentolamine or phenoxybenzamine 121

IV. DISCUSSION 126

A. WHOLE ORGAN PERFUSION 127

B. NORADRENALINE ANALYSIS OF PLACENTA AND UMBILICAL CORD 128

C. FLUORESCNECE MICROSCOPY OF UMBILICAL TISSUE 131

D. EFFECT OF DRUGS ON NORADRENALINE UPTAKE 134

1. Comparison of the extraneuronal uptake process in non-innervated tissues to that in innervated tissues 134

2. The mechanism and function of extraneuronal uptake 140

E. ISOLATED TISSUE PREPARATION 144

1. General pharmacology of the isolated tissue preparation 144

2. Effect of cocaine on responses to agonists 149

3. Protection experiments 151

F. EVALUATION OF THE "COCAINE PARADOX" 153

	Page
V. SUMMARY AND CONCLUSIONS	166
VI. BIBLIOGRAPHY	170
APPENDIX A	206
APPENDIX B	207
APPENDIX C	209

LIST OF TABLES

Table		Page
I.	Responses to tyramine in perfused placenta preparation	67
II.	Noradrenaline induced tyramine response	70
III.	Effect of cocaine on the response to noradrenaline in the isolated perfused placenta	71
IV.	Noradrenaline content of human umbilical cord and placenta	72
V.	Noradrenaline uptake	90
VI.	Noradrenaline loss from tissue after previous incubation in 40 μg/ml noradrenaline-Krebs for 15 minutes	91
VII.	Effect of temperature on noradrenaline uptake	93
VIII.	Effect of cocaine on the uptake of noradrenaline by the umbilical artery	94
IX.	Effect of various agents on the uptake of noradrenaline by the umbilical artery	96
X.	Approximate ED_{50}'s for agonists	100
XI.	Effect of cocaine on noradrenaline	103
XII.	Effect of cocaine on tyramine	105
XIII.	Effect of cocaine on 5-hydroxytryptamine	107
XIV.	Effect of cocaine on histamine	109
XV.	Effect of cocaine on KCl	111
XVI.	Effect of phentolamine on responses to noradrenaline, tyramine and 5-hydroxytryptamine	114
XVII.	Effect of propranolol on responses to noradrenaline, tyramine and 5-hydroxytryptamine	117
XVIII.	Effect of noradrenaline on induced-tyramine response	120
XIX.	Effect of DPTC on responses to 5-hydroxytryptamine, noradrenaline and tyramine	123
XX.	Cocaine protection against receptor blockade by phentolamine or phenoxybenzamine	125

LIST OF FIGURES

Figure		Page
1.	Placental perfusion apparatus	44
2.	Isolated tissue apparatus	46
3.	Trihydroxyindole procedure. Formation of fluorescent fluorophores from noradrenaline	50
4.	Apparatus for determining effect of drugs on noradrenaline uptake by umbilical arteries.	58
5.	The histochemical reaction between noradrenaline and formaldehyde	61
6.	Noradrenaline dependence of the response to tyramine in the perfused whole placenta preparation	66
7.	Effect of cocaine on the response to noradrenaline in the perfused whole placenta preparation	69
8.	Responses to tyramine in the isolated umbilical artery	98
9.	Responses to oxytocin, angiotensin and acetylcholine in the isolated umbilical artery	99
10.	Effect of cocaine on the responses to noradrenaline in the isolated umbilical artery	102
11.	Effect of cocaine on the induced tyramine response in the isolated umbilical artery.	104
12.	Effect of cocaine on the responses to 5-hydroxytryptamine in the isolated umbilical artery	106
13.	Effect of cocaine on the responses to histamine in the isolated umbilical artery	108
14.	Effect of cocaine on the responses to KCl in the isolated umbilical artery	110
15.	Effect of phentolamine on responses to noradrenaline, tyramine and 5-hydroxytryptamine	113
16.	Effect of propranolol on the responses to noradrenaline and tyramine	115

Page

Figure

17. Effect of propranolol on responses to 5-hydroxytryptamine 116

18. Effect of noradrenaline on the tyramine responses in the isolated umbilical artery and the isolated guinea-pig vas deferens 119

19. Effect of DPTC on the responses to 5-hydroxytryptamine, noradrenaline and tyramine 122

20. Effect of cocaine on receptor blockade produced by phentolamine and phenoxybenzamine 124

21. Configuration relationships between phenethylamine, D(-)noradrenaline, and (-) cocaine 161

LIST OF PHOTOGRAPHS

Photograph		Page
1.	Transverse section of normal umbilical cord to show umbilical artery	74
2.	Rat heart section to show adrenergic nerve endings	75
3.	The section as in photograph 2, but after water treatment	75
4.	Rabbit aorta to demonstrate nerve endings in adventitia. Tissue frozen and sectioned 5 minutes after death	77
5.	Rabbit aorta as in photograph 4, but frozen and sectioned 1 hour after death	77
6.	Blood vessels in rabbit heart detected in tissue which was frozen and sectioned 1 hour after death of animal	78
7.	Blood vessel, with adrenergic nerve endings, rabbit spleen after storage overnight in Krebs.	80
8.	Blood vessel, from rabbit spleen as in photograph 7, but incubated with 10 μg/ml noradrenaline for 30 minutes.	80
9.	Unilocular fat cells from rabbit spleen	81
10.	Unilocular fat cells from rabbit spleen incubated with 10 μg/ml noradrenaline for 30 minutes	81
11.	Umbilical artery after incubation with 40 μg/ml noradrenaline for 30 minutes	82
12.	Same section as illustrated by photograph 11, but after water treatment	82
13.	Umbilical artery elastic fibers after incubation with 40 μg/ml noradrenaline for 30 minutes	83
14.	Same section as illustrated by photograph 13, but after water treatment	83
15.	Umbilical artery after incubation with 10 μg/ml noradrenaline for 30 minutes	85
16.	Sequential section to that illustrated by photograph 15 but heat treated with no paraformaldehyde	85
17.	Umbilical artery pretreated with 10^{-4}M parnate for 30 minutes and then incubated with 40 μg/ml noradrenaline for a further 30 minutes	86

"Hallo!" said Piglet, "What are you doing?"
"Hunting," said Pooh.
"Hunting what?"
"Tracking something," said Winnie-the-Pooh very mysteriously.
"Tracking what?" said Piglet, coming closer.
"That's just what I ask myself. I ask myself, What?"
"What do you think you'll answer?"
"I shall have to wait until I catch up with it, " said Winnie-the-Pooh.

A. A. Milne

I. INTRODUCTION

I. INTRODUCTION

A. GENERAL INTRODUCTION

Cocaine has been claimed to produce an increase in the sensitivity of the effect of adrenaline, noradrenaline and 5-hydroxytryptamine on the blood vessels of the human placenta and umbilical cord (von Euler, 1938; Eliasson and Åström, 1955; Åström and Samelius, 1957; Gulati and Kelkar, 1971). In other vascular tissues the mode of action of cocaine in producing supersensitivity to noradrenaline is generally thought to be by blockade of neural uptake of noradrenaline and thus increasing the local concentrations of noradrenaline in the region of the receptors. In tissues which are not innervated this mechanism is not available to cocaine. It is commonly thought that the blood vessels of the placenta and umbilical cord do not receive innervation, although this view has recently been questioned (Ten Berge, 1962a, 1962b, 1963; Jacobson and Chapler, 1967, 1968; Fox and Jacobson, 1969a).

The problem of the innervation of the placenta and umbilical cord and the mode of action of cocaine in this system is examined in this introduction first by a discussion of the structure and function of the placenta, a review of publications describing the responses of this organ to pharmacological agents, and finally a discussion of the possible mode of action of cocaine.

B. STRUCTURE AND FUNCTION OF THE PLACENTA AND UMBILICAL CORD

The human placenta is of the hemochorial type (Grosser, 1927), the foetal blood being separated from the maternal blood by the three

layers of the maternal membrane (endothelium, connective tissue and epithelium). At full term the umbilical cord with its arteries and one vein enters the "chorionic plate"; the umbilical vein transports blood to the foetus under a pressure of about 30-60 mm Hg (Reynolds, 1954) and the umbilical arteries return blood to the placenta at approximately 90/55 mm Hg (Nyberg and Westin, 1958). Dawes (1964) has estimated that 55% of the combined ventricular output of the foetal heart leaves by way of the two umbilical arteries which anastomose prior to entering the placenta (Arts, 1961; Panigel, 1962; Crawford, 1962). Placental circulation in monkeys has been well described by Ramsey (1960), using the angiographic technique, and the "Physiological theory", which supposes that maternal blood enters the intervillous space of the placenta in spurts and diffuses into relatively localized areas where it circulates about the placental villi before leaving by nearby veins, is generally accepted. It is thought that this circulation pattern also occurs in the human placenta but may differ in other mammals.

C. POSSIBLE MECHANISMS OF POST-PARTUM CHANGES

Several important changes occur in the fetal cardiovascular system at birth:

1) Interruption and eventual obliteration of the umbilical vessels.

2) Closure and eventual obliteration of the ductus venosus.

3) Closure of the foramen ovale and final separation of the two atria by a definite septum.

4) Gradual constriction of the ductus arteriosus and eventual obliteration and transformation into a fibrous structure.

5) Dilation of the pulmonary arteries.

6) Elimination of the placental circulation and redistribution of blood flow and vascular resistance.

Little is known by which mechanisms the ductus arteriosus (D.A.), ductus venosus (D.V.) and umbilical arteries (U.A.) close at birth; several mechanisms, however, have been proposed and these can be summarised briefly:

1) pO_2. Assali et al. (1968) have demonstrated in the lamb that a critical pO_2 of 55 mm Hg is necessary before a decrease in D.A. flow occurs. An increase in pO_2 of perfusing fluids causes strong contractions of isolated placental and cord vessels in both animals and man (Schmitt, 1922; Haselhorst, 1929) and anoxia causes vascular dilation (Haselhorst, 1929).

2) Temperature. Decreased temperature causes constriction of the umbilical arteries (Haselhorst, 1929); however, in certain ruminants, the vessels contract into the abdomen and in these cases temperature changes cannot be the primary cause of the closure.

3) Mechanical Mechanisms. A Bayliss-type response (due to stretch) has been suggested by Shepherd (1968) as being responsible for the closure of the cord vessels.

4) Amine Mechanisms. Podegra (1967) has suggested that 5-hydroxytryptamine may be involved in the closure of the cord vessels at birth because the smooth muscle is very sensitive to this substance.

Brzezinski *et al*. (1962) have shown that placental 5-hydroxytryptamine levels increase during pregnancy whilst those of monoamine oxidase (MAO), found in placenta and amniotic fluid by Luschinsky and Singher (1948), decrease. Koren et al. (1965, 1966a, 1966b) also postulate an important function for 5-hydroxytryptamine in the initiation of parturition.

Gokhale *et al*. (1966) have suggested a possible function of histamine in constricting human umbilical vessels, and Kahlsson and Rosengren (1968) have suggested a similar role for histamine in the rat.

Born *et al*. (1955), Kovalcik (1963), Ehinger *et al*. (1968), Boréus *et al*. (1969), and Aronson *et al*. (1970) have suggested that noradrenaline, adrenaline and acetylcholine may be implicated in the closure of the D.A. and D.V. The studies of Assali *et al*. (1968), however, do not entirely support this hypothesis.

5) Bradykinin. A role of this kinin in human umbilical artery closure has been postulated by Gokhale *et al*. (1966), Eltherington *et al*. (1968), and Melmon *et al*. (1968). However, Bor and Guntheroth (1970) found that closure of the D.A. and U.A. can occur in the apparent absence of bradykinin.

6) Oxytocin. Oxytocin, a vasoconstrictor in the placental circulation and umbilical arteries, can be used for the bioassay of oxytocic activity (Krejci, personal communication). It is, however, questionable whether maternal oxytocin could reach the foetal circulation in sufficient amounts to close the umbilical artery because of the high concentration of placental oxytocinase (Polacek *et al*. 1970).

7) Angiotensin. Angiotensin has been shown by Gauteri and Mancini (1967) to constrict the placental vasculature, and renin production

has also been demonstrated in the chorion of the placenta.

8) <u>Prostaglandins</u>. Karim (1966, 1967), Karim and Devlin (1967), and Hiller and Karim (1968) have demonstrated the presence of prostaglandin E_1 [PGE_1], PGE_2, PGF_1 and PGF_2 in the amniotic fluid and umbilical cord of humans. PGE_2, F_1 and F_2 are capable of constricting the umbilical artery and it has been suggested that these may have a function in closing the umbilical artery when the foetus has been expelled since earlier in gestation the vessels do not contain these contractile prostaglandins. Karim (1970) has also used PGG_2 for inducing therapeutic abortion.

Despite our increasing knowledge of this area, we are still unable to say what mechanisms are involved in the closure of the three foetal systems: the ductus arteriosus, the ductus venosus and the umbilical arteries. In the case of the D.A. and D.V. it has been stated that adrenergic nerve fibres are associated with these organs (Ehinger <u>et al</u>., 1968; Boréus <u>et al</u>., 1969), but this does not necessarily imply that noradrenaline is the mediator for closure (Assali <u>et al</u>., 1968). Pearson (1970) has demonstrated that a small bundle of nerve fibres pass from the anterior and posterior vagal trunks between the layers of the hepatogastric ligament. These fibres pass to the junction of the umbilical vein and ductus venosus walls. Nerve fibres pass to this junction and to the proximal portion of the umbilical vein, nerves from the celiac plexus are present and thus it is possible that both sympathetic and parasympathetic nerves could function in this region.

D. INNERVATION OF THE UMBILICAL CORD AND PLACENTA

Galen (130-200 A.D.) stated that the human placenta and cord lacked innervation and since this time the accuracy of this statement has been questioned on numerous occasions. Lack of innervation in both the human and certain other animals has been supported by the work of Schott (1836), Valentin (1837), Kolliker (1879), Goenner (1906), Bucura (1907, 1908), Schmitt (1922), Stöhr (1928, 1932a, 1932b, 1938), Spivack (1943), but the positive findings of Fossati (1905, 1906, 1907), Argaud (1922), Mabuchi (1924), Dancz (1931), Ooi (1934), Scalzo (1939, 1940), Coujard (1952), Kernbakh (1959) and TenBerge (1962a,1962b,1963) have contradicted their findings. Ueda (1931) also suspected from pharmacological studies that the human placenta and umbilical cord must be innervated.

More recently, Jacobson and Chapler (1967), using the methylene-blue immersion technique of Miller and Kasahara (1963), found numerous nerve trunks in the human placental villi. The small vessels within the human amnion and the chorion were also found to be accompanied by fine nerve fibres (1968). Similar results were then obtained when the human umbilical cord was examined using this method by Fox and Jacobson in 1969(a) and a plexus of nerves near the walls of the umbilical cord vessels was found in the Wharton's Jelly. Fox and Jacobson thus proposed that vasomotor responses in the umbilical cord vessels could be mediated by a neural mechanism. Fox and Jacobson (1969b) later showed that there was also a rich plexus of nerves in the placental system of the two sub-human primates, *Macacu phillipnesis* and *Gralago crossicaudatus*. Lauweryns (1969) studied the osmium-iodide nerve-staining technique of Champy-Coujard as used by TenBerge (1962a, 1962b, 1963).

and concluded that it lacked the required selectivity needed for nerves; similarly the methylene-blue technique used by Fox and Jacobson (1968, 1969a, 1969b) and Jacobson and Chapler (1967) lacked sufficient selectivity. The nerve trunks found by these workers probably represent collapsed chorionic capillaries or strands of connective tissue. Margot Roach (personal communication) believes that the nerve fibres demonstrated by various people may in fact be muccopolysaccharide strands stained by the histological technique employed. Ammorozo, who has performed extensive studies on foetal circulation (reviewed by Barclay et al., 1944) also believes that the recent reports of nerves in the umbilical cord were likely to be artifacts (personal communication). He has only ever seen nerve-like structures in two ox umbilical cords.

Pearson and Sauter (1969) found that innervation was fairly rich within the first few inches of the cord but beyond this point the nerves quickly decreased in number. "End-nets" were only present as far as four inches from the umbilicus. Nadkarni (1970), however, although failing to demonstrate nervous tissue in the human umbilical cord using silver stains, claimed to have demonstrated myelinated nerves in the mid-zone of the cord by using electron microscopic methods. Leeson and Leeson (1965) were unable to detect nerve fibres associated with the blood vessels of the umbilical cord of the rat using electron microscopic methodology. Perhaps species differences may partly explain the differences found in studies of umbilical innervation. Thus, Hülsemann (1971) using electron microscopic methods failed to detect any extrafoetal innervation of the guinea-pig umbilical cord. Lachenmayer (1971) utilizing light and fluorescence microscopic studies detected

adrenergic fibres only in the vicinity of the umbilicus (guinea-pig, man, mouse and rabbit). Ellison (1971), by employing a thiocholine technique, revealed extrafoetal acetylcholinesterase-positive nerves in the full-term human umbilical cord. Nerve bundles passed from the foetus into the cord and split into a plexus and beaded nerve terminals were found 15-20 cm from the foetus. Although some of these endings touched the media of the vein, he thought it unlikely that they could detect chemical changes in the venous blood. Ellison did not detect the "sub-amniotic neural plexus" described by Fox and Jacobson (1969a) and extends the opinion that the plexus was probably due to intercellular substance stained with methylene blue, thus supporting Lauweryns (1969). Ellison also examined the extrafoetal vitelline vessels of the rat and found a plexus around the blood vessels and thus it is possible that closure of the extrafoetal vitelline vessels of newborn rats is under neural control; however, nerves cannot be implicated in closure of the vessels of the allantoic part of the umbilical cord of the rat since these vessels are not innervated.

Read and Burnstock (1970) have detected adrenergic nerves in various foetal organs by incubating pieces of such tissues in MAO-resistant α-methyl noradrenaline. This technique increases the content of neuronal catecholamine and facilitates the detection by fluorescence histochemistry, of nerves with subthreshold noradrenlaine. Using the same technique, Walker and McLean (1971) failed to detect adrenergic nerves in the human umbilical cord.

The absence of adrenergic nerves is further substantiated by the lack of noradrenaline in the umbilical cord tissues (Davidgnon, 1964).

Lachenmayer (1971), however, has detected very low concentrations (0.03 - 0.06 μg/g) of noradrenaline in whole umbilical cord homogenates using samples obtained from the guinea pig and the rabbit. It is possible, in the absence of information as to what portions of the umbilical cord were used, that samples may have included the nerve containing proximal end of the umbilical cord. Noradrenaline and adrenaline have been detected in the amniotic fluid of the human by Zuspan (1970), but this probably originates from the foetal urine. Coraboeuf _et al_. (1970) have found that acetylcholine could be released from chick embryo heart even before the heart was innervated. The placenta has been shown to contain acetylcholine (Chang and Wong, 1933), cholinesterase (Werle and Hartman, 1952) and MAO (Luschinsky and Singher, 1948). MAO has also been detected in the umbilical cord by Gennser and Studwitz (1969). If cholinergic and adrenergic nerves are not present in this tissue then it is interesting to speculate as to the function of these mediators and enzymes. It is unlikely that these substances are directly related to an intrinsic innervation since it would seem that innervation would have developed by term. Nerves which contain noradrenaline can be detected in foetal tissues at approximately 12 weeks or even earlier if the tissues are incubated with noradrenaline (Read and Burnstock, 1970). The MAO patterns may reflect the developing adrenergic innervation of the vessels, just as the development of MAO activity during ontogenesis of the chick is regarded as reflecting the appearance of sympathetic innervation in this animal (Ignarro and Shideman, 1968). Alternatively it may be suggested that nerves are present earlier in development but degenerate

toward term; this is unlikely since Ehinger et al. (1968) were unable to detect adrenergic nerves in the umbilical cord of 20-24 week old human foetuses, although nerves were detected in the ductus venosus portion.

With regard to the innervation of the placenta and umbilical cord one can conclude that although generally accepted as being devoid of innervation there is a lack of complete agreement to this statement. Even though the human placenta and umbilical cord may lack innervation, the vessels have been shown to be sensitive to the action of neurohormones and other drugs.

E. PHARMACOLOGY OF PLACENTA AND UMBILICAL CORD

Many perfusion experiments have been performed to discern the effects of various drugs on the placental vaculature. The mechanism of action of adrenaline, noradrenaline, acetylcholine, 5-hydroxytryptamine and histamine on the placental vasculature has captured the interest of numerous investigators (von Euler, 1938; Dornhorst and Young, 1952; Eliasson and Åström, 1955; Åström and Samelius, 1957; Gauteri and Cuichta, 1962, 1963, 1964; Gauteri and Mancini, 1964). Many drugs can cross the placental barrier and thus their effects on the placental vasculature is important for it is possible that when such drugs are administered to pregnanat women they may disturb the homeostatic conditions of the placenta and exert serious effects on the foetus. Evidence suggests that noradrenaline can in fact cross the placenta and affect the haemodynamics of the foetus (Beard, 1962; Sandler et al., 1963).

Schmitt (1922) was one of the earlier workers to test the effects of drugs on the placental vasculature. He found that histamine, posterior pituitary extract, or barium chloride contracted the isolated human placental vessels, amyl nitrite produced a dilation, and adrenaline had no effect. He believed the inactivity of adrenaline to be evidence that the human placenta is not adrenergically innervated.

Kosakoe (1927) reported that adrenaline produced constriction of the vessels in perfused human placentas. Ueda (1931) confirmed this effect of adrenaline and later (1932) found that vessels in placentas removed prior to the sixth month of pregnancy were unresponsive to adrenaline and pilocarpine, but that vasculature in placentas removed after this time was constricted by adrenaline and dilated by pilocarpine.

Eliasson and Åström (1955), by perfusing via one umbilical artery, found that D(-)adrenaline and D(-)noradrenaline caused a marked constriction in placentas with long (at least 8 cm) umbilical cords. A lesser degree of constriction was noticed with placentas with short cords or where the cord was absent. It is thus possible that the principal site of action of adrenaline and noradrenaline is on the umbilical cord vessels. They also found that 5-hydroxytryptamine and histamine caused vasoconstriction whereas acetylcholine had variable effects.

Nyberg and Westin (1957) found that adrenaline, noradrenaline, and an increase in oxygen tension all resulted in an increase in perfusion pressure.

Åström and Samelius (1957) found that 5-hydroxytryptamine caused a constriction of the placental vessels in perfusion experiments. Similarly, Panigel (1959) found that adrenaline, noradrenaline, 5-hydroxytryptamine and dihydroergotamine produced vasoconstriction and that the action of 5-hydroxytryptamine was inhibited by BOL (d-2-bromolysergic acid butanolamide). The placental vasculature as a whole appeared to be less responsive than umbilical vessels.

Goerke *et al.* (1961) developed a technique whereby both foetal and maternal sides of the placenta are perfused. An increase in O_2 and CO_2 tensions produced constriction and dilation respectively. Histamine and 5-hydroxytryptamine caused constriction of the umbilical artery, but the action of noradrenaline and adrenaline was unclear. Krantz *et al.* (1962) and Hamrin *et al.* (1971) have developed a similar but somewhat more sophisticated procedure which has not yet been used, to study the responsiveness of placental and umbilical vessels to drugs.

In situ placental perfusion experiments have been conducted by Dawson and Robson (1940) on cats and dogs. Adrenaline was found to cause an increase in perfusion pressure. Dornhurst and Young (1952), using the *in vivo* perfusion of guinea pig and rabbit placentas, found that adrenaline and noradrenaline may act upon maternal and placental circulations directly rather than by production of uterine contractions. In either case, the result would be an impairment of placental blood flow with a subsequent production of foetal asphyxia.

The results of placental perfusion investigations are by no means in agreement either quantitatively or qualitatively. Even when rigid control of the artificial environment is maintained, and careful techniques practised, the simulation of a true maternal-foetal relationship is very difficult. This may explain the difference in the nature of the effects of certain drugs on the placental vasculature. Takenaka (1963) noted contractions to both tyramine and noradrenaline in isolated spirally cut umbilical arteries of the human, but the responses to histamine and 5-hydroxytryptamine were much greater.

It is interesting to speculate on the effects of cocaine on the adrenergic mechanisms in this vascular system. von Euler (1938) showed that cocaine could potentiate the effects of adrenaline in the isolated perfused human placenta. Eliasson and Åström (1955) also found that cocaine enhanced the actions of 5-hydroxytryptamine, noradrenaline and adrenaline in a few instances. If the placental and umbilical vasculature system is not innervated then it would seem possible that cocaine cannot be potentiating the action of adrenaline and noradrenaline by blocking neural uptake. Somlyo _et al_. (1965) were, however, unable to confirm the potentiation effect of cocaine on noradrenaline responses in helical strips of the human umbilical artery. Dyer (1969, 1970a, 1970b) found that cocaine potentiated 5-hydroxytryptamine responses in spirally cut sheep umbilical blood vessels but had only a small effect on responses to noradrenaline and adrenaline. Gulati and Kelkar (1971), using a technique similar to that described by Gokhale _et al_. (1966) for perfusion of the human umbilical artery, have found that cocaine could augment the responses to tyramine and noradrenaline, but not those to adrenaline,

acetylcholine and 5-hydroxytryptamine.

F. POSSIBLE MECHANISMS OF ACTION OF COCAINE

Cocaine has long been known to augment the pressor action of certain catecholamines (Fröhlich and Loewi, 1910). There would appear to be two general types of mechanism whereby cocaine can cause potentiation of adrenergic responses:

1. Inhibition of catecholamine inactivation (blockade of uptake; inhibition of MAO; inhibition of catechol-o-methyl-transferase [COMT]).

2. Direct effect of cocaine on the effector tissues.

The evidence for both these types of mechanism is discussed below.

1. Inhibition of inactivation of catecholamines by cocaine.

(a) Blockade of MAO and COMT

Gaddum and Kwiarowski (1938) suggested that cocaine caused supersensitivity to adrenaline by the inhibition of MAO. However, the concentration of cocaine required to inhibit MAO (Philipot, 1940) is 10^3 to 10^6 times those required to produce supersensitivity. Cocaine can also potentiate cobefrine which is not metabolised by MAO (Jang, 1940). More recently the use of potent MAO inhibitors have indicated that little or no supersensitivity can be produced following MAO inhibition (Furchgott, 1955).

Inhibition of COMT has also been implied, but cocaine can produce supersensitivity to amines not metabolised by COMT (Trendelenberg

et al., 1962b), and while inhibition of COMT can produce a prolongation of responses to noradrenaline and adrenaline there is little increase in magnitude of the response. There is no evidence that cocaine can inhibit COMT and this possibility can be dismissed.

(b) Inhibition of catecholamine uptake

It was postulated by Burn (1932) that catecholamine storage sites existed which could be replenished from the extracellular fluid adjacent to the nerve ending. The experiments of Whitby et al. (1960), Dengler et al. (1961), and Muscholl (1961) have demonstrated that cocaine can inhibit noradrenaline uptake into sympathetic nerve endings. Also a correlation between blockade of noradrenaline uptake and the sensitization caused by cocaine has been demonstrated by Macmillan (1959), Whitby et al. (1960), Furchgott et al. (1963), Hardman et al. (1965), Trendelenburg (1966), Draskoczy and Trendelenburg (1968). The "uptake theory" of supersensitivity (Trendelenburg, 1966) has thus been generally accepted to explain the action of cocaine. Many other drugs, besides cocaine, have also been shown to compete with noradrenaline for uptake into these storage sites, and Iversen's work (see review, 1967) compares these compounds and their relative efficiency in blocking catecholamine uptake. Further support for this theory is provided by the observation that cocaine does not sensitize sympathetically denervated tissues (Langer et al., 1967) and that its sensitizing action appears to be stereospecific for agents that are taken up into the adrenergic nerve endings. Further support for the suggestion that cocaine acts by inhibition of catecholamine uptake has been provided by Tye et al. (1967).

2. Direct Action on the Effector Cell

Although the "uptake theory" is fairly widely accepted, some evidence exists that cocaine may sensitize adrenergic responses by a mechanism(s) other than that of an inhibition of uptake into the nerve ending. Before the advent of the "uptake theory", Clark (1937) suggested that supersensitivity may be due to an altered affinity of the receptors for agonists. This suggestion was later supported by Maxwell et al. (1959, 1961). Although Trendelenburg (1965) showed that D(-)noradrenaline was taken up more readily than L(+)noradrenaline in the cat nictitating membrane, it was later shown by Draskoczy and Trendelenburg (1967, 1968a) that there was no difference in uptake between D(-) and L(+)noradrenaline in the isolated perfused rabbit heart. Iversen et al. (1971b) demonstrated, by use of an in vivo double isotope technique, that in the rat and mouse heart (-)noradrenaline was taken up significantly more rapidly than (+)noradrenaline, but no stereochemical selectivity was found for noradrenaline uptake in guinea pig hearts. Obviously, considerable species differences may exist in this phenomenon (cf. Jarrott, 1970). Maxwell (1965a) and Reiffenstein (1968) have pointed out that potentiation of agonist responses in isolated tissues is inadequately explained by the "uptake theory". Maxwell et al. (1966) also found no parallelism between the degree of block of uptake and the degree of supersensitivity to noradrenaline.

Varma and McCullough (1969) found that, by cold storage of smooth muscle, it was possible to dissociate the supersensitivity to noradrenaline caused by cocaine from inhibition of H^3-noradrenaline uptake. Based on the doubtful hypothesis that most of the uptake and

storage of noradrenaline is in sympathetic nerves (Stromblad and Nickerson, 1961; Wolfe *et al.*, 1962) and that cocaine inhibits the uptake of noradrenaline by the sympathetic nerves (Maxwell *et al.*, 1968), Varma reasoned that, if supersensitivity is due to inhibition of noradrenaline uptake, cocaine should not sensitize a nerve-free tissue to noradrenaline. Nerve-free smooth muscle was obtained by cold-storage of (6-8°C for 7 days) rabbit aortic strips, rabbit splenic capsules and rat nictitating membranes, and the effect of cocaine on the noradrenaline responses was thus determined after degeneration of intrinsic sympathetic nerves. Ambache (1946), and Kosterlitz and Lees (1964) had observed that whilst cold storage leads to degeneration of intrinsic nerves the responsiveness of the tissue was only slightly altered. It was found that whilst (±)-H^3-norepinephrine uptake was significantly reduced by storage in the cold, cocaine could still potentiate the responses of stored smooth muscle to noradrenaline. Varma suggested that cocaine may be acting postsynaptically, perhaps on the adrenergic receptors, and thus enhancing noradrenaline responses. Varma's experiments do not necessarily contradict Trendelenburg's "uptake theory" because this was based mainly on observations on nictitating membrane and heart under more physiologic conditions. Graefe and Trendelenburg (1970) report that cold storage of the muscle neither abolishes the neuronal uptake of noradrenaline nor the ability of cocaine to impair this uptake (both parameters were, however, reduced) and they thus conclude that there is no reason to doubt the causal relation between impairment by cocaine of neuronal uptake and ensuing supersensitivity to D(-)noradrenaline. Graefe and Trendelenburg's conclusions probably differ

from those of Varma because Varma assumed that decreased accumulation of exogenous H^3-noradrenaline is a measure of neuronal uptake, whereas Graefe and Trendelenburg state that a decreased accumulation of H^3-noradrenaline in whole tissue is not necessarily indicative of a failure of the uptake across the nerve membrane.

Langer *et al*. (1967), as previously stated, showed that cocaine did not sensitize sympathetically denervated tissues; however, Ozawa and Sugawara (1970) found that cocaine potentiated noradrenaline responses after denervation of the guinea pig vas deferens. Possibly tissue and/or species differences may exist which would help to explain some of these apparent contradictions. Ozawa and Sugawara conclude that the supersensitivity of the isolated guinea pig vas deferens to noradrenaline is mainly due to impairment of noradrenaline uptake into the adrenergic nerve endings but that a postsynaptic mechanism, namely an action on the receptor and on the muscle membrane, cannot be excluded. In a denervated tissue the amine uptake mechanism is, of course, inoperative (Birmingham, 1970); thus cocaine potentiation, if it occurs, must be due to a postsynaptic effect rather than blockade of uptake.

In support of the theory that cocaine causes supersensitivity to noradrenaline by directly altering the receptor mechanism is the recent work of Innes and Karr (1971). They suggest that the combination of cocaine with cellular sites alters the adrenoceptive receptor sites and thus causes the observed supersensitivity. Support for this hypothesis is provided by the observation that phentolamine can protect the α-adrenergic receptor against cocaine potentiation (Innes and Karr, 1971). In this technique the tissue was tested for cocaine potentiation,

in a protected tissue, when the adrenergic blocking action of phentolamine had been washed out.

An interesting observation reported by Wakade et al. (1970) was that supersensitivity to 5-hydroxytryptamine after postganglionic sympathetic denervation, decentralisation (pre-ganglionic denervation), and cocaine treatment may be partly due to the impairment of the uptake process and partly due to changes in the postsynaptic membrane. Cocaine increased the 5-hydroxytryptamine response of the denervated nictitating membrane of the dog, but had no effect on the noradrenaline response. Cocaine treatment led to a further increase in sensitivity of the decentralised membrane to noradrenaline; however, in such a preparation the uptake mechanism is still intact but the capacity of the adrenergic nerve endings for the uptake may have been reduced after decentralisation. Certainly the evidence suggests that cocaine potentiates the 5-hydroxytryptamine response partly by changes in the postsynaptic membrane.

Fleming (1971) concluded that supersensitivity of denervated rat diaphragm had two components: a marked highly specific supersensitivity to cholinergic agents and a small, nonspecific supersensitivity to potassium. It was suggested that postjunctional supersensitivity of denervated smooth and cardiac muscle resembled the second component (nonspecific supersensitivity) and that supersensitivity in smooth and cardiac muscle may be the result of changes in membrane or coupling mechanisms rather than from invocation of the nebulous concept of an increase in the number of receptors. Possibly cocaine may also induce similar changes in smooth muscle.

The work of Kalsner and Nickerson (1969a,b,c,d) and Kalsner (1971), using the oil immersion technique to eliminate the diffusion of drugs

from rabbit aortic strips, has suggested that the increased amine response after cocaine is more likely due to a direct action on effector cells. The evidence can be summarised thus:

1) Cocaine has been found to potentiate methoxamine, an amine which has negligible affinity for the nerve membrane transport system, and which cannot be inactivated by other processes (COMT or MAO).

2) Histamine responses are enhanced by cocaine although it has no effect on the rate of inactivation of this amine; 5-hydroxytryptamine, however, is not potentiated (rabbit aorta) although uptake is decreased by cocaine.

3) Responses to noradrenaline are potentiated by cocaine even when strips are stored for 10 days in the cold, or at 37°C for 28 hours to permit degeneration of the sympathetic nerve terminals. This supports the results obtained by Varma and McCullough (1969) described previously.

4) Phenylephrine responses are potentiated by cocaine after all known intraneuronal mechanisms of amine disposition are eliminated by pretreatment with reserpine and a MAO inhibitor; changes in solubility of the drugs after oil-immersion occurs is not known and this, of course, may complicate interpretation of the results.

Kalsner and Nickerson's work is also dependent on the hypothesis (as demonstrated by the use of the oil immersion technique) that neuronal uptake and storage can play a relatively minor role in terminating the action of noradrenaline in the rabbit aorta.

Leszkovsky and Tardos (1968) also showed that cocaine potentiated the responses to isopropylnoradrenaline (INA) in cat spleen strips (this amine is not taken up by adrenergic nerves (Hertting, 1964; Iversen, 1964; Hardman _et al_., 1965). Similarly, Davidson

and Innes (1968) have also demonstrated supersentivity to INA in the presence of cocaine using isolated cat spleen strips; INA acts on α-excitatory receptors in this tissue (Bickerton, 1963). Provided that INA is not taken up into nerve terminals, this observation supports the hypothesis that cocaine has an effect on the postsynaptic membrane in order to cause INA potentiation. Uptake of INA by the guinea pig trachea has been shown by Foster (1969); however, this probably represents extraneuronal uptake and is not blocked by cocaine.

Reiffenstein (1968) demonstrated, using cat spleen strips, that while cocaine did not increase the rate of contraction of these tissues it did increase the strength of contraction induced by noradrenaline. "Uptake theory" predicts that an increase in effective concentration should have increased contraction rate. An explanation was given in terms of Paton's rate theory of drug action (1961) and an allosteric alteration of the α-receptor by cocaine. Further support for this theory is suggested by Reiffenstein and Nakatsu (1968) and Nakatsu and Reiffenstein (1968) who show that cocaine cannot be acting only by blocking noradrenaline uptake. They suggest that the increased utilization of available α-receptors in the presence of supramaximal doses of noradrenaline after phenoxybenzamine treatment is due to an increased efficacy, again possibly due to an allosteric alteration of the α-receptor by cocaine.

Bevan and Verity (1967) prepared a surgically denervated preparation by stripping the adventia from the rabbit aorta; on this preparation cocaine did not change the slope, or shift the dose-response curve but did significantly increase the maximal contraction of the

strips. Using innervated strips, cocaine was found to cause a shift of the dose-response curve and thus they concluded that the supersensitivity produced was due to both a pre-synaptic mechanism and a direct component of action on the smooth muscle.

The reported instances where cocaine has potentiated the effects of noradrenaline, adrenaline, and 5-hydroxytryptamine on placental or umbilical vessels would seem to suggest that cocaine must be causing the supersensitivity by acting on the effector cell receptor mechanism. However, it is known that smooth muscle itself can take up noradrenaline in the absence of nerve and the possibility exists that cocaine can cause potentiation by interference with this mechanism.

G. EXTRANEURONAL UPTAKE OF NORADRENALINE

Iversen (1963) found that perfusion of a medium containing a low concentration of noradrenaline, 0.2 μg/ml, resulted in accumulation, by the heart, of this amine. Adrenaline, 0.5 μg/ml, was also found to be accumulated in the isolated perfused rat heart (Iversen, 1965a) by the same mechanism as that of noradrenaline; this observation confirmed the original suggestion of Stromblad and Nickerson (1961) which was that adrenaline and noradrenaline compete for a common uptake site in tissues. This neuronal uptake mechanism was designated <u>Uptake</u>$_1$ (U_1)(Iversen, 1965b) in order to distinguish it from <u>Uptake</u>$_2$ ($\underline{U}_2$) which occurred when there were higher concentrations of the amines in the perfusion fluid, >1 μg/ml. The properties of $\underline{U}_1$ were found to be distinct from those of $\underline{U}_2$ (Iversen, 1965b). The histochemical work of Ehinger <u>et al</u>. (1968) suggests that

this uptake is both intraneuronal and extraneuronal. Extraneuronal uptake had already been suggested to occur in denervated tissues (particularly the salivary gland) where COMT and MAO had been inhibited (Stromblad and Nickerson, 1961; Andén et al., 1963; Fischer et al., 1965; Potter et al., 1965; Iversen et al., 1966; Eisenfeld et al., 1967). Fluorescent histochemical studies demonstrating extraneuronal uptake, have also been performed (Gillespie and Hamilton, 1966; Hamberger, 1967; Hamberger et al., 1967; Ehinger and Sporrong, 1968). Clarke and Jones (1969) found that this accumulation was independent of the presence of postganglionic adrenergic nerves by destruction of sympathetic terminals with 6-hydroxydopamine, the noradrenaline being mainly located in smooth muscle cells. However, interpretation of experiments using 6-hydroxydopamine are complicated by our lack of knowledge regarding the possible non-selective actions of this compound. Recently, Haeusler (1971) has demonstrated both early pre- and post-junctional effects of 6-hydroxydopamine.

Fischer et al. (1965) demonstrated that, following chronic sympathetic denervation of rat salivary gland, there was a greatly diminished ability to bind and retain 3H-noradrenaline; however, a residual capacity did remain and this was shown to be due to an extraneuronal site of noradrenaline uptake.

Eisenfeld, Axelrod and Krakoff (1967) later found that certain adrenergic blocking agents decreased noradrenaline accumulation into extraneuronal sites of the rat heart after inhibition of neuronal uptake with cocaine. Metaraminol, which is a potent inhibitor of U_1, has little effect on this extraneuronal accumulation. Normetanephrine, however

caused a large decrease in the amount of noradrenaline accumulated by these extraneuronal sites. These workers suggested that the proposed extraneuronal transport mechanism may either regulate access to the receptor or may be part of the receptor mechanism itself. Alternatively, the transport mechanisms may function to increase access of noradrenaline to the intracellular enzymes that metabolize the catecholamine. As mentioned, α-adrenergic blocking agents could decrease this extraneuronal uptake and phenoxybenzamine was particularly effective. The membrane ATPase inhibitor, ouabain, had little or no effect.

Draskoczy and Trendelenberg (1968b, 1970) found that in the denervated nictitating membrane, extraneuronal uptake of noradrenaline was demonstrable at a concentration of 1 μg/ml racemic noradrenaline-3H. Extraneuronal uptake was decreased by metanephrine and by phenoxybenzamine, increased after COMT and/or MAO inhibition, and unchanged by cocaine or reserpine. The rate of uptake of different amines is in the following order: isopropylnoradrenaline > adrenaline > noradrenaline.

Using the Falck fluorescent histochemical technique, Gillespie and co-workers (Gillespie et al., 1966; Gillespie and Hamilton, 1966, 1967; Avakian and Gillespie, 1967; Gillespie, Hamilton and Hosie, 1967, 1970; Gillespie and Muir, 1970) have provided similar results for several tissues. Species differences exist (possibly related to different levels of MAO and COMT in tissues) and the uptake was primarily into smooth muscle cells although other structures also increase in fluorescence. The noradrenaline was rapidly lost from the tissue with a half time of 7-8 minutes. Their results suggested that this extraneuronal uptake

was a threshold phenomenon occurring when the noradrenaline concentration was between 10^{-6} and 10^{-5} g/ml. On the basis of these results, Gillespie et al. (1970) classified three types of amine uptake mechanisms:

Uptake$_1$: into nerve endings operating at a threshold of 10^{-8} g/ml and saturating at 5 x 10^{-7} g/ml. This uptake was stereospecific, reserpine resistant, and sensitive to cocaine, DMI or metaraminol.

Uptake$_2$: (NOT the same as Iversen's U_2) operates at a threshold of 10^{-6} g/ml and saturates at 5 x 10^{-5} g/ml. This uptake is reserpine-sensitive and stereospecific and probably represents granular (or vesicle) uptake. It is NOT influenced by cocaine, DMI or metaraminol.

Uptake$_3$: (probably equivalent to Iversen's U_2) operates at a threshold of 10^{-5} g/ml; probably not saturable, non-stereospecific, reserpine- and cocaine-insensitive, inhibited by o-methylated metabolites of adrenaline, noradrenaline and normetanephrine and by phenoxybenzamine; occurs in denervated tissues and represents uptake into smooth muscle or cardiac muscle.

Although extraneuronal uptake is inhibited by phenoxybenzamine, this inhibition was not related to α-receptor blockade as it is possible to dissociate the concentrations necessary for blockade of extraneuronal-uptake and α-receptor blockade (Gillespie, 1968). Lowering the temperature, and ouabain treatment also cause a decrease in the uptake of noradrenaline. However, it was not known whether the uptake is an active process or not; tissue to medium ratios close to three were demonstrated on the basis of fluorescent histochemical studies.

Lightman and Iversen (1969) found that extraneuronal uptake of

noradrenaline into cardiac muscle cells could occur at physiological concentrations of noradrenaline and thus the extraneuronal uptake into cardiac muscle is not a threshold phenomenon. Thus uptake$_2$ (cf. Iversen) does not abruptly become activated at certain high critical concentrations of external catecholamine but operates throughout the range of external catecholamine concentration. Lightman and Iversen (1969) used noradrenaline levels from 0.5 μg/ml and upwards and found accumulation at this concentration, when both MAO and COMT were inhibited. It has not, however, been shown whether uptake into smooth muscle is a threshold phenomenon, but it would appear likely that it is similar to the uptake into cardiac muscle and is thus not a threshold process. The failure of Gillespie to demonstrate fluorescence in smooth muscle cells with noradrenaline concentration less than 10 μg/ml is probably due to the insensitivity of this method for detecting low levels of diffuse fluorescence and also due to the metabolic activity of MAO and COMT in the tissues.

Jacobowitz and Brus (1971) suggest that both fibroblasts and vascular smooth muscle constitute major sites for extraneuronal uptake and metabolism of catecholamine. However, since Jarrott (1970) has demonstrated the existence of many species differences in the uptake and metabolism of noradrenaline, such findings emphasize that caution must be exercised in extrapolating results concerning noradrenaline uptake determined in one organ of one species to other organs and other species. This fact is illustrated by the recent work of Burnstock et al. (1971) who have compared extraneuronal uptake in rabbit ear artery to that in the non-innervated tissue of human umbilical

artery and chick amnion, using fluorescence histochemistry. Their results demonstrate that accumulation of noradrenaline within non-innervated smooth muscle cells is observed histochemically after exposure to much lower concentrations of noradrenaline (10^{-7} g/ml) than in sympathetically innervated smooth muscle cells, where accumulation is observable with noradrenaline at 10^{-5} g/ml. Also, the uptake in non-innervated tissue is not inhibited by phenoxybenzamine, normetanephrine or cold, although some inhibition was apparent at noradrenaline concentrations lower than 10^{-4} g/ml. After inhibition of COMT by catechol however, the accumulation of noradrenaline in innervated smooth muscle closely resembles that in non-innervated smooth muscle. It is possible that low levels of COMT and/or MAO may lower the levels of extraneuronal uptake.

Iversen (1971) suggests that "$\underline{\text{uptake}_2}$" may have an important role in the inactivation of circulating catecholamines and that "$\underline{\text{uptake}_2}$"- and -metabolism might be the predominant mechanism for noradrenaline inactivation in tissues such as vascular smooth muscle, in which the density of sympathetic innervation, in certain parts of the tissue, is very low.

It would appear that if noradrenaline effects are potentiated by cocaine in the human umbilical artery and if cocaine does not influence the smooth muscle uptake of noradrenaline in this tissue, then one can conclude, providing no nerves are present, that cocaine induced supersensitivity is due to an effect on the muscle which causes an alteration of the α-receptor mechanism of the effector cells.

H. ALLOSTERISM AND DRUG RECEPTORS

1. Allosteric changes and cocaine action

It would appear that there is some justification for proposing that the mode of action of cocaine in producing supersensitivity to noradrenaline cannot be explained solely by the ability of cocaine to block noradrenaline uptake into the nerve ending. A possible allosteric alteration of the α-receptor has been proposed as a mechanism by which cocaine may alter the postsynaptic membrane (Reiffenstein and Nakatsu, 1968; Reiffenstein, 1968; Nakatsu and Reiffenstein, 1968; Innes and Karr, 1971). The conclusions of certain other workers also suggest a possible change in the α-receptor (Maxwell *et al.*, 1966; Bevan and Verity, 1967; Varma and McCullough, 1969; Kalsner and Nickerson, 1969b).

2. General theories of allosterism, conformational changes, and cooperativity

The evidence for the existence of allosteric interactions was obtained initially from enzyme studies by Monod *et al.* in 1963 and a detailed model was proposed by the same authors in 1965.

The term "allosteric" was introduced to describe a site on the enzyme, distinct from the active site at which the substrate binds, and which is complementary to the structure of a regulatory molecule referred to as the allosteric effector. One function of the allosteric effector is to modify the properties of the active site through a change (allosteric transition) in the kinetic parameters of the protein. Since 1963 the term allosteric has been used to include the phenomenon of cooperativity. Thus, allosterism now describes both the effects of

ligand binding at a non-catalytic site on catalytic efficiency, and also the ability of ligand binding of the active site to affect the binding of additional molecules, of the same ligand, to other active sites.

Many enzyme and protein systems show complexities in ligand binding. In particular, it is often noted that the ligand saturation curve does not follow the hyperbolic relationship characteristic of Langmuir-type absorption, but follows a sigmoid relationship. This sigmoid curve suggests that at least two molecules of ligand bind to the protein and that the binding of the first ligand molecule facilitates the binding of subsequent molecules. Such a cooperative interaction exists, for instance, in the well-investigated oxygen-haemoglobin system (Adv. Protein Chemistry, 1964).

Conformational changes induced by various substrates on the enzymes have also been explained by the "induced-fit" theory of Koshland (1963). Koshland (1958, 1959, 1962, 1963, 1964) noted that "lock and key" hypothesis of enzyme action, originally proposed by Emil Fischer in 1894, could not explain all parameters of enzyme systems and he thus initiated investigations which led to the "induced-fit" theory. Thus the active site of an isolated crystalline enzyme does not necessarily need to have a morphology that is complementary to that of the substrate. The substrate, in combining with an enzyme, induces a change in its conformation; this results in an enzymically active orientation of the catalytic groups.

The basic features of Koshland's hypothesis are that, (a) small molecules induce conformational changes on binding to the enzyme, (b) a

precise orientation of catalytic groups is needed to cause reaction and (c) substrates induce the proper alignment of catalytic residues whereas nonsubstrates do not. There is abundant evidence that conformational changes may occur in enzymes under physiological conditions (Yankeelou and Koshland, 1965; O'Sullivan and Cohn, 1966; Mehler and Cusic, 1967; Adelman et al., 1968; Morse and Horecker, 1968; DeLuca and Marsh, 1967; Hammes, 1968a, 1968b), although it has been suggested that such changes are usually quite small (Lumry and Biltonen, 1969). The induced-fit theory has been extended to explain drug action; a drug may intereact with an enzyme, or a noncatalytic protein, at a particular binding modifier (Cennamo, 1968) or allosteric site (Monod, 1966). This site would constitute part of the receptor, and may be visualized as possessing an elastic morphology which can be deformed or altered and then returned to its original form after deformation (Grisolia, 1964). Such a receptor has been described as "dynamic" by Bloom and Goldman (1966). Koshland's and Monod's theories are not necessarily mutually exclusive as the conformational change may be due to an allosteric process (Monod, 1966).

Koshland et al. (1966) and Koshland and Neet (1968) have adapted the "induced-fit theory" in order to explain cooperative effects - the phenomenon that binding of one ligand molecule somehow accelerates binding of subsequent ones. Thus binding of the first ligand to a polymeric protein (which may be an enzyme containing the receptor) induces a conformational change in one of its subunits. The resulting stabilization allows stronger binding of succeeding molecules.

Analogous theories have recently been suggested to explain the

conformational alteration of the protomers that constitute the macromolecules (Changeux *et al*. 1967; Wyman, 1967, 1968; Noble, 1969). In 1964, Belleau introduced the "macromolecular perturbation theory" which is really an application of the "induced-fit theory" to certain classes of drugs. The basic points are that: (a) Drug-receptor interactions very often follow the law of mass-action, mainly in relation to competitive antagonism. (b) Formation of the drug-receptor complex is attended by change in free-energy, whence it is deduced that this complexation takes place with chemical modification in the receptor due to changes in vibrational and electronic energies. (c) Conformational changes in proteins that act as receptors convert them from inactive species to ones capable of catalysing reactions with substrates. Depending on whether the drug produced specific or non-specific conformational changes, it would behave as an agonist or an antagonist. Support for this theory comes from the work of Portoghese (1965), Belleau and Lavoie (1968), and Kay and Robinson (1969).

3. Allosteric changes and the cholinergic receptor

The existence of allosteric mechanisms appears to be indicated for the action of acetylcholine and its antagonists at the muscarinic receptor (reviewed by Moran and Triggle, 1970; and Triggle, 1971), as an allosteric interaction accomodates, in a satisfactory manner, the action of atropine and related ligands. Also, following the possible isolation of an acetylcholine receptor, Miledi *et al*. (1971) found that prolonged exposure to acetylcholine reduced the binding of bungarotoxin by as much as 80%. This evidence suggests that the desensitization

phenomena may involve some change in the conformation of the receptor molecules.

Several investigations have also shown that acetylcholinesterase may be an allosteric enzyme (Changeux *et al*. 1968; Kitz *et al*. 1970; Meunier and Changeux, 1969; Wombacher and Wolf, 1971).

4. Allosteric changes and the adrenergic receptor

Besides the allosteric effect suggested for cocaine and the evidence that cocaine does in fact alter the postsynaptic membrane there is also other evidence suggesting adrenergic allosteric changes.

Maxwell *et al*. (1961) suggested that methylphenidate alters the affinity of the receptor substance for amines and this results in the supersensitivity seen after methylphenidate administration. Also, Maxwell (1965b) has speculated that guanethidine and derivatives cause supersensitivity to noradrenaline by changing the configuration of the receptor "surface" in such a way as to improve hydrogen bonding or ion pair formation with selected amines and hence to enhance the affinity of the receptor for the amine. Varma (1966) proposed that the changes in sensitivity to noradrenaline, which occur in the cat nictitating membrane after denervation, arise from a qualitative change in the α-receptors.

Patil (1969) speculated that postganglionic denervation caused qualitative changes in the α-receptor and altered its steric configuration. Patil supported this claim by showing that the isomeric ratio [ratio between (−) and (+) noradrenaline] was markedly different from that

obtained after cocaine treatment. These results can also be considered as reflecting a change in the α-receptor after cocaine treatment; the change being different from that occurring after denervation. This possibility has been suggested by Patil et al. (1971).

Nickerson and House (1958) found that prolonged receptor blockade by phenoxybenzamine produced supersensitivity of the cat nictitating membrane to noradrenaline and adrenaline. They found that cats, pretreated in vivo with phenoxybenzamine, developed supersensitivity to noradrenaline at the same rate as those cats which had been denervated; this suggested to Nickerson and House that the absence of an effect of the normal mediator at the α-receptor was possibly a major factor in causing sensitzation. Kurahashi and Shibata (1971) have speculated that bretylium increases the sensitivity of the effector cell to K^+ or tyramine by a direct action on the postsynaptic membrane of rabbit aortic strips.

I. EFFECT OF COCAINE ON SMOOTH MUSCLE CALCIUM

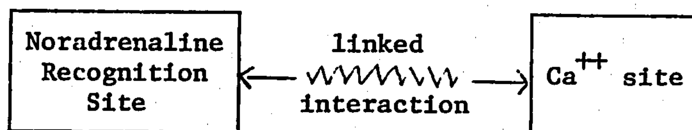
Shibata et al. (1971) have speculated that potentiation of noradrenaline responses by cocaine in rabbit aortic strips results from the increased entry of extracellular Ca^{++}. Shibata et al. demonstrated that cocaine failed to potentiate the noradrenaline response in a Ca^{++} free medium; Mn^{++} and Co^{++} which are reported to inhibit Ca^{++} flux (Hagiwara and Nakajima, 1966; Geduldig and Junge, 1968; Shibata, 1969a) prevented cocaine potentiation; cocaine also potentiated the contraction produced when Ca^{++} is added to a Ca^{++}-free, high potassium medium. A

common mechanism to explain supersensitivity in terms of an increased membrane permeability had already been suggested by Carrier and Shibata (1967) and Shibata (1969a). Cocaine has been shown to affect calcium binding (Hurwitz et al., 1962; Daniel, 1964). Daniel and Wolowyk (1966) proposed that, in uterine segments of the rabbit, cocaine caused labilization of calcium loosely bound in the membrane. The comparitively high concentrations of cocaine used in this study (0.1 to 2.0 mg/ml) caused contraction of the uterus, presumably by virtue of the Ca^{++} labilization; cocaine did not appear to act by releasing known neurotransmitters or by directly affecting the receptors for these transmitters. An action on oxytocin receptors was also unlikely because oxytocin failed to produce a contraction in progesterone-dominated uteri at 24°C although these tissues were still responsive to cocaine. An effect of cocaine on Ca^{++} mechanisms may also explain the muscle contraction induced in the rat vas deferens by cocaine (Vohra, 1969); cocaine and tetracaine (Vohra, 1970a) and xylocaine and procaine (Vohra, 1970b). Greenberg and Innes (1968), using cat spleen strips, suggested that noradrenaline is less dependent on extracellular Ca^{++} for contraction in the presence of cocaine and this may be due to release, by cocaine, of Ca^{++} from an intracellular store. Kasuya and Goto (1968), using the rat vas deferens, found that cocaine potentiated responses to noradrenaline, angiotensin, acetylcholine and potassium; they suggest that cocaine may act by increasing the availability of Ca^{++}, which may be liberated from bound stores or induced to permeate across the membrane by agonists. Although this evidence suggests that cocaine lacks selectivity in its potentiating effect, it was found in this

adrenergic system, and the denervated rat vas deferens (Kasuya *et al.* 1969), that the degree of potentiation of noradrenaline responses was significantly greater than that observed with other agonists, and thus part of the noradrenaline supersensitivity may be based on an agonist-specific mechanism. Nakatsu (1968) suggested that cocaine primes a Ca^{++} pool which is utilized specifically by the α-receptor thus enhancing the noradrenaline response.

J. CALCIUM AND THE α-RECEPTOR

The possible role of cocaine in mobilizing Ca^{++} and thus potentiating noradrenaline-induced contractions of smooth muscle might seemingly diminish the importance of the original postulate that cocaine causes an allosteric-like change in the α-adrenergic receptor. However, this conclusion may be incorrect as Moran *et al.* (1970) have provided evidence that suggests a Ca^{++} mobilization site existing in the membrane that is linked specifically to a noradrenaline recognition site. The noradrenaline recognition site and the Ca^{++} binding site may be functionally inseparable such that Ca^{++} is not made available to the excitation-contraction-coupling process until the noradrenaline recognition site is activated. Thus, there may be a linked process similar to that postulated by Wyman (1968). An allosteric arrangement may thus be indicated:



This suggests that the catecholamine-α-receptor interaction initiates the mobilization of a proportional number of Ca^{++} ions to generate a proportional muscle response. Belleau (1967), on theoretical grounds, has placed Ca^{++} displacement as a prominent function for agonist interaction at the α-receptor.

K. COCAINE AND THE α-RECEPTOR

Whether cocaine interacts with a calcium mobilization site is not known. The action of cocaine in producing potentiation would appear to be nonselective because, in various tissues, it also potentiates the actions of other agonists such as K^{+}, acetylcholine, angiotensin, histamine and 5-hydroxytryptamine. However, it would appear to produce a greater degree of potentiation towards noradrenaline (Kasuya and Goto, 1968) as a greater leftward shift of the dose-response curve was found with noradrenaline than with the other agonists. This is also true when supersensitivity is produced by other mechanisms (Fleming, 1971). Perhaps cocaine has some selectivity towards the response mediated by the α-adrenergic receptor. Vohra (1969) has suggested that cocaine may act as a mixed sympathomimetic (acting directly on the α-receptor and also causing noradrenaline release) on the rat vas deferens; however, cocaine failed to protect α-adrenergic receptors against phenoxybenzamine blockade. Cliff (1968) found that cocaine caused contraction of the reserpine-treated rat vas deferens in which no tissue catecholamines were detectable by Falck's fluorescence technique. Cocaine also caused contractions in denervated rat vas deferens. These results

can be explained in terms of the Ca^{++} releasing capacity of cocaine. Vohra (1969), however, found that although the rat vas deferens was contracted by cocaine the guinea pig vas deferens did not respond. This fact is interesting because Janis and Triggle (1971) have shown that, following DMPEA blockade of the α-receptor, the half time for recovery to noradrenaline in the rat vas deferens was 23 minutes, the same as that obtained for the rabbit aorta. The half time for recovery in the guinea pig vas deferens, however, was 75 minutes, similar to that for the rabbit vas deferens. These results may reflect differences at, or very close to, the noradrenaline binding site of the α-receptor. Whether such differences could explain the reason for the contraction of the rat vas deferens by cocaine and the lack of response in the same tissue of the guinea pig is not, however, known. van Rossum (1965) and Furchgott (1970) have also suggested that differences in α-receptors may exist.

Although agonists on smooth muscle probably all produce the same end-result, that is, an elevation of the concentration of free calcium in the intracellular fluid near the contractile protein and then, ultimately, an alteration of the interaction of myosin and actin causing contraction of the muscle cell, they may do so by different mechanisms. For instance, the greater selectivity of SKF-525A towards potassium induced contractions than towards those of other agonists in the rabbit aortic strip preparation has been recently demonstrated by Kalsner _et al_. (1970), and similar results have been observed in the same preparation and in the rat vas deferens by Triggle (personal communication). It was concluded by Kalsner _et al_.

that SKF-525A specifically inhibits the movement of extracellular and/or loosely bound calcium to the contractile elements of vascular smooth muscle in response to membrane depolarization by potassium. SKF-525A was found to have little effect on the mobilization of firmly bound calcium by noradrenaline but might, however, interfere partially with replenishment of this pool from extracellular sources.

L. AIMS OF THE RESEARCH

Evidence exists that cocaine may have an effect on the post-synaptic membrane which could influence the noradrenaline response. However, it has been shown quite clearly that cocaine can influence noradrenaline uptake into the nerve ending and this also can explain, to some degree, the potentiating effect of cocaine on the noradrenaline response. In an innervated tissue it would thus appear that cocaine may have at least two modes of action which could explain its influence on adrenergic mechanisms. These actions are difficult to differentiate.

The human umbilical cord is, classically, considered to be non-innervated and thus if cocaine can influence the noradrenaline response in this system it could not be doing so by virtue of blocking neuronal uptake of noradrenaline. However, recent observations question the "classical" view that the umbilical cord is non-innervated and thus we cannot suppose that the action of cocaine involves post-synaptic effects alone unless it has first been demonstrated that the human umbilical cord is devoid of adrenergic innervation. This problem has been investigated by noradrenaline analysis and fluorescent microscopy.

An MAO inhibitor has also been used in conjunction with the above techniques in order to determine whether the activity of tissue MAO may influence noradrenaline distribution in this system.

Nerves need not be present in a tissue for noradrenaline uptake to occur; thus extraneuronal "uptake" (accumulation) of noradrenaline has been observed in many tissues of various species. Possibly cocaine may influence noradrenaline responses by affecting this extraneuronal accumulation. It is therefore necessary to study the effect of cocaine on the extraneuronal accumulation of noradrenaline in the human umbilical artery tissue. The characteristics of this accumulation of noradrenaline in isolated human umbilical arteries has also been compared to those of innervated systems.

A postsynaptic action of cocaine on calcium binding and calcium movements in the effector cell has also been shown and such an action may be the common denominator in explaining this and, perhaps, other supersensitivity phenomena. It would appear that cocaine may have a nonspecific action which could influence the action of other smooth muscle agonists whose action is unrelated to affinity for the α-receptor. This problem was investigated by determining the effect of cocaine on other agents which influence smooth muscle contraction in the human umbilical artery and thus determine the specificity of the action of cocaine.

This investigation therefore sets out to answer the following questions in relation to the manner in which cocaine may influence noradrenaline responses in the isolated human umbilical artery:

1) Is the human umbilical artery innervated by adrenergic nerves?

2) Can noradrenaline be accumulated by human umbilical artery tissue?

3) If noradrenaline accumulation can occur in the umbilical artery, is it influenced by cocaine?

4) Is the potentiating action of cocaine specific to noradrenaline or can it also influence the action of other smooth muscle agonists?

II. METHODS

II. METHODS

A. PERFUSION OF WHOLE PLACENTA

Human placenta were obtained within 15 minutes of delivery from the Department of Obstetrics at the University of Alberta Hospital and immediately 200 ml of heparinized Krebs-Ringer (10 I.U./ml) at 38°C was injected into the placenta via an umbilical artery to flush blood from the placenta and help prevent clot formation. The placenta was then transported to the laboratory in a container of preheated Krebs-Ringer solution (see Appendix A) at 38°C and placed in the perfusion apparatus which consisted of two plexiglass chambers. The lower chamber served as a water jacket and through this, water at 40°C was circulated by means of a Heto thermoregulator, thus maintaining the aerated Krebs in the upper chamber at 37°C.

The upper perfusion chamber had an entrance tube for Krebs solution, the Krebs being maintained at 40°C in a warming coil enclosed by a water jacket and the Krebs source for the coil being supplied from a heated reservoir (see Figure 1).

One umbilical artery was cannulated about 5 cm from the surface of the placenta; a cord of longer than 5 cm is often extremely spiralled offering constrictions and other obstructions which make the perfusion of the placental vasculature difficult. The other umbilical artery was ligated. A polyethylene cannula was inserted into the umbilical vein at the same level as the arterial cannula in order to measure outflow and determine fluid loss by edema. Failure of 40% of the

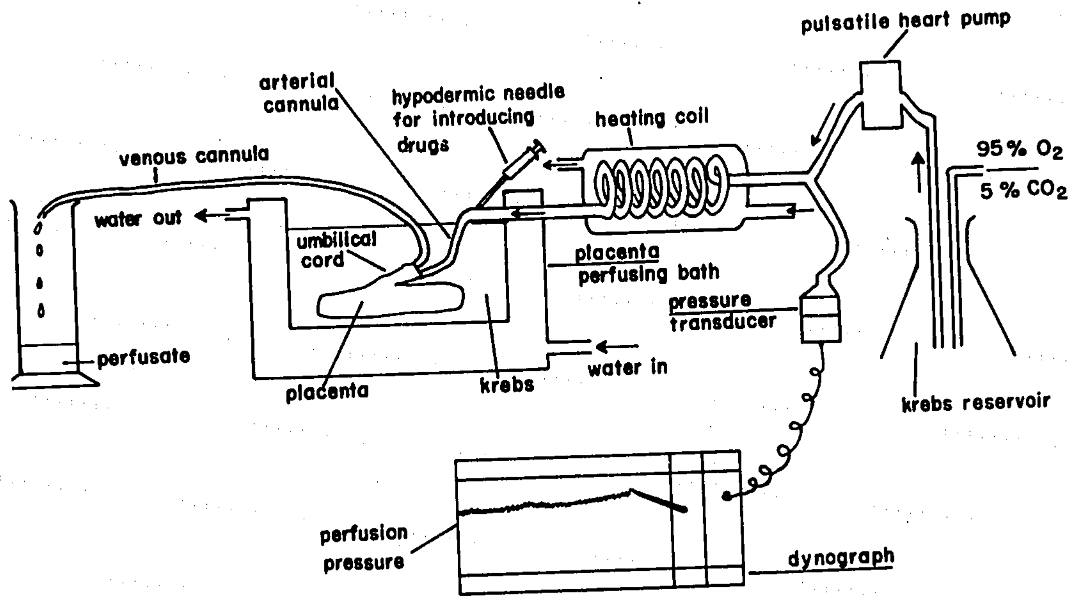


Figure 1. Placental perfusion apparatus

preparations (50 of 120) has been noted. This has usually resulted either from unstable baseline perfusion pressures, probably due to blood clot formation, or from an inability to obtain a venous outflow, possibly due to structural damage of the placenta.

Perfusion pressure was maintained at 40-50 mmHg (Nyberg and Westin, 1958) and the flow of Krebs maintained at 10-20 ml/min by a constant output Harvard Pulsatile "Heart" Pump; pressure was recorded via a pressure transducer (Statham). Drug injections were made into a rubber connection tube adjacent to the artery cannula; the drugs were dissolved in 0.5 - 1.0 ml of 0.9% saline . A list of the drugs employed can be found in Appendix B. Perfusion was normally started within 30 minutes of delivery; the Krebs solution used for perfusion was aerated (95% O_2 - 5% CO_2) and modified with 2% polyvinylpyrrolidone (PVP) to maintain osmotic pressure and prevent edema.

B. PREPARATION OF ISOLATED UMBILICAL ARTERY STRIPS

The apparatus for experiments involving isolated strips of umbilical artery is illustrated in Figure 2. This apparatus allows the bathing solution to be prewarmed while passing through a glass coil beneath a muscle bath whose working volume is 10 ml. The water circulating around the muscle bath and through the glass warming coils was maintained at 37°C by continuous circulation of water from a reservoir and the water temperature was thermostatically controlled. The Krebs solution was maintained in a reservoir and supply to the muscle bath was controlled by use of pinchcocks. Both the Krebs

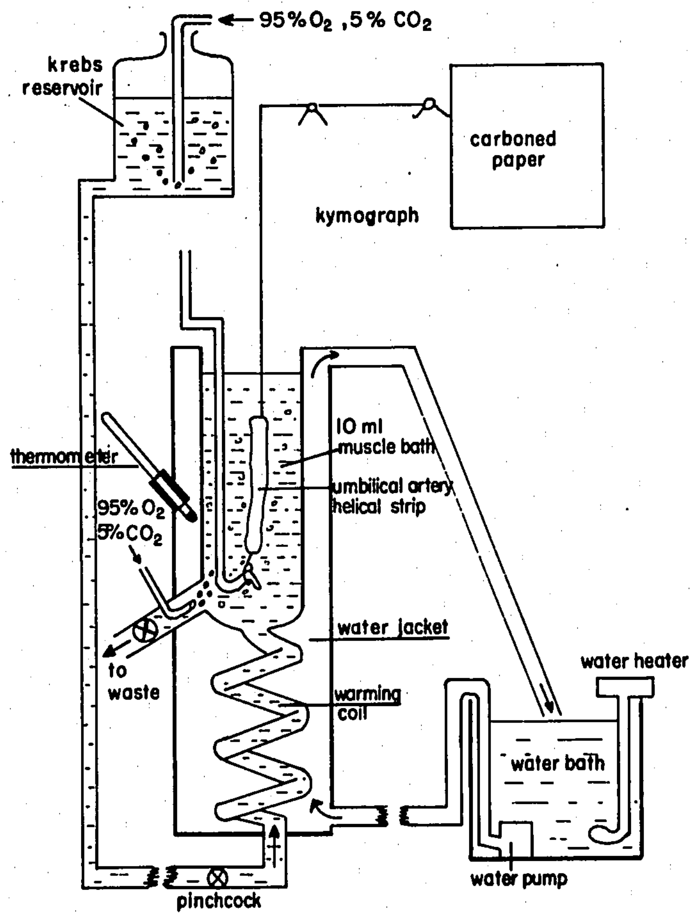


Figure 2. Isolated tissue apparatus

reservoir and the muscle bath were aerated with 95% O_2 and 5% CO_2. the muscle bath being aerated via a fine polyethylene tubing (Clay-Adams PE 20) passed through the drainage tube.

Isolated strips of umbilical artery were obtained by the method described by Furchgott and Bhadrakom (1953). The artery was freed of connective tissue and cut spirally to provide strips 3-4 cm long and 2 mm wide. The strips were mounted in the muscle baths under a tension of two grams and allowed to equilibrate for $1\frac{1}{2}$ hours during which time the strips were washed with fresh Krebs solution every 10 minutes. Contractures were recorded isotonically and magnified x 7 with a frontal writing lever on a carbon kymograph recorder with Palmer kymograph extension. The speed of the paper was maintained at either 0.0425 mm/sec or 0.0850 mm/sec.

Drugs were added to the bath using a 1/4, 1/2 or 1 ml syringe at a maximum dose of 0.2 ml. The drugs were washed out of the bath either when a maximal height of contraction had been reached or 8 minutes after administration, whichever was sooner. Antagonists and cocaine were added to the bath usually 5 minutes before the agonist; a further 8 minutes was then allowed for tissue response.

Where indicated, the ED_{50}, dose of drug required to give 50% of the maximal tissue response, for each drug was calculated by testing the tissue to a range of four concentrations of the drug. Dose-response curves, with a sufficient number of points, are very difficult to produce in this tissue because of the prolonged nature of tissue relaxation. The use of dose-response curves in these type of experiments would thus be influenced by tissue desensitization and fatigue and would greatly

affect the results. A similar rationale has been employed by Graefe *et al*. (1971) and Trendelenburg (1971) for the study of supersensitivity caused by impairment of intraneuronal mechanisms of amine inactivation in the cat nictitating membrane.

The Krebs-Ringer solution used for these experiments was modified by the addition of 10^{-5}M disodium ethylenediamine tetra-acetic acid (EDTA) in order to decrease autoxidation of sympathomimetic amines (Furchgott, 1960; Nedergaard, Vagne and Bevan, 1968). This treatment was effective for protection of sympathomimetic amines for short exposure times of about 5 minutes (Graefe and Trendelenburg, 1970).

C. CATECHOLAMINE ANALYSIS

A multitude of procedures have been reported for the estimation of catecholamines in biological tissues, many of which are based on the selective absorption of the catecholamine by aluminum oxide, a technique originally devised by Shaw in 1938. This is followed by oxidation of the noradrenaline in the eluate to a fluorescent trihydroxyindole (THI) derivative, or condensation of the noradrenaline in the eluate with ethylenediamine. The THI procedure was adopted, with the modifications similar to those described by Anton and Sayre (1962), because of the greater specificity reported for this technique (von Euler, 1961; Valk and Price, 1956), as compared with the ethylenediamine condensation.

The fluorescence of oxidation products of epinephrine in alkaline solution was first observed by Loew in 1918 and was further studied by Paget (1930). This was used as the basis of a fluorometric analysis

technique (Gaddum and Schild, 1934; Richter, 1937) and then adapted into the THI procedure by Ehrlen (1948) and Lund (1949a,b,c). It has been found that fluorescent THI derivatives are formed when the catecholamines, in a slightly acid solution, are oxidized by iodine, or potassium ferricyanide, and then subjected to a strongly alkaline solution containing an antioxidant. The production of noradrenolutine from noradrenaline is shown in Figure 3 (Harrison, 1963). Although the method used resembles that described by Anton and Sayre (1962), several changes were introduced and thus the procedure and reagents are described in detail below.

1. Reagents (Highest Purity). Fisher primary standard reagents were used for the preparation of reagents and Krebs solution (except where otherwise stated).

a) Aluminum oxide (Woelm Neutral Activity Grade 1). Aluminum oxide (100 grams) was treated with 2N HCl (500 ml) at 90°C for 45 minutes with constant stirring using a magnetic stirrer combination with hot plate. After 45 minutes the stirring was discontinued and the mixture allowed to settle for 1½ minutes. The yellow supernatant fluid was then discarded along with the finer particles of aluminum oxide. The remaining aluminum oxide was then washed twice with 2N HCl (250 ml) at 70°C for 10 minutes each. The supernatant with the finer aluminum oxide particles were discarded on each occasion. Finally the aluminum oxide was stirred with 2N HCl (500 ml) at 50°C for 10 minutes. The aluminum oxide was then washed repeatedly with 200 ml portions of distilled water until the supernatant had a pH of 3.4, the finer

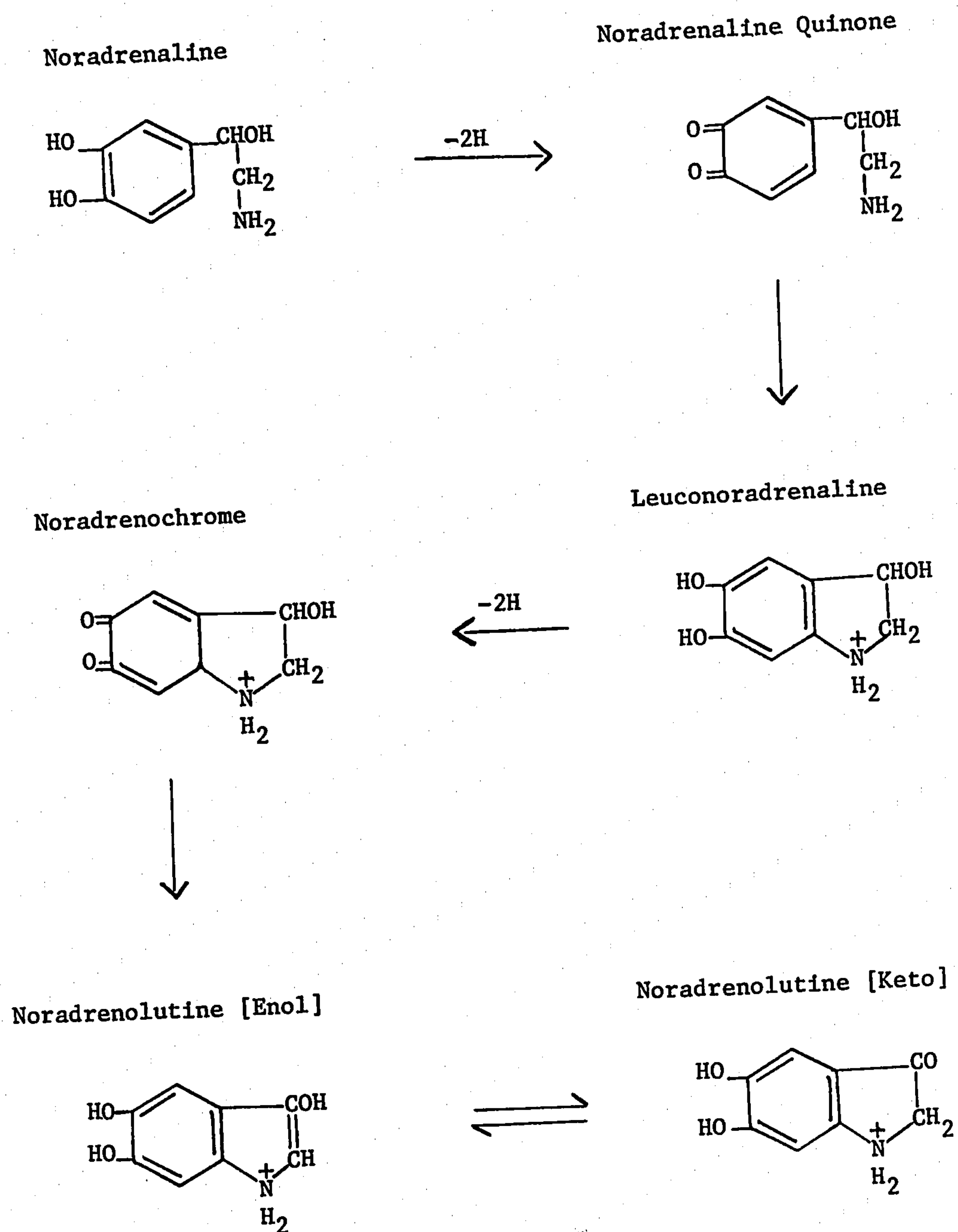


Figure 3. Trihydroxyindole Procedure
Formation of fluorescent fluorophores from noradrenaline.

particles were discarded each time. The aluminum oxide was transferred to an evaporating dish and heated for 1 hour at 120°C and then for 2 hours at 200°C. Finally the dried powder was stored in a dessicator at room temperature.

b) 0.4M Perchloric acid. Perchloric acid was used to homogenize the tissue samples. Other agents, such as trichloracetic, hydrochloric, nitric and sulphuric acids, have been found to be less suitable (Anton and Sayre, 1962; Bertler *et al.*, 1958a, 1959b).

c) 0.2M Acetic acid. Acetic acid was used for elution of the catecholamines from the alumina. Anton and Sayre (1962) suggest the use of 0.05M perchloric acid but it was found, in our laboratory, that the use of 0.2M acetic acid gave more consistent results.

d) 0.5M Tris Buffer, pH 9.0. This solution was prepared (60.7 g/1 of tris[hydroxymethyl]aminomethane) and brought to pH 9.0 by use of 2N hydrochloric acid.

e) Acetate Buffer, pH 7.0. This buffer was made by dissolving disodium ethylenediaminetetraacetate dihydrate (EDTA)(37.2 grams) with sodium acetate (136.1 grams, 1 mole) and making up the volume to one liter. The pH was then adjusted to 7.0 by adding 5N NaOH.

f) Iodine. Iodine (1.27 grams) was dissolved in absolute ethanol (100 ml) and stored in a dark bottle.

g) Alkaline sulphite. This solution was made just prior to use by diluting a 25% aqueous solution of anhydrous sodium sulphite (1 ml) with 5N sodium hydroxide (9 ml).

h) Distilled water. All solutions used were made with glass distilled water which was prepared as follows: glass distilled water

was re-distilled in a pyrex all glass distillation apparatus in the presence of EDTA (1 gram/liter). The distillate was then re-distilled in the same apparatus. This triple glass distilled water was then employed for the preparation of all solutions.

2. Preparation of Samples

Prior to analysis all tissue samples were kept below 0°C by the use of liquid nitrogen. The tissue sample, usually weighing about 0.5 grams, was first homogenized in 0.4M ice-cold perchloric acid (5 ml) using a glass tissue grinder which was semi-submerged in an ice bath. The homogenate was then centrifuged at 3,000xg in a clinical centrifuge (International Clinical Centrifuge - Model CL) for 5 minutes. One ml of the clear supernatant was transferred to a tube containing alumina (500 mg), Tris buffer (7 ml) and 1 ml of a known concentration of noradrenaline, usually 10^{-7}g/ml, to act as an internal standard; the pH of this mixture was between 8.2 and 8.4. The tube was then sealed with a rubber stopper and shaken for 15 minutes on a Techni-Lab Rotator (Model 715) shaker, centrifuged for 1 minute and the supernatant aspirated off and discarded. The alumina was washed with glass distilled water for 1 minute; this water was then aspirated off and discarded. The alumina was shaken for 15 min with 0.2M acetic acid (3 ml) to elute the catecholamine and the mixture was then centrifuged for 1 minute.

3. Formation of the Fluorescent Trihydroxyindole derivatives

a) Procedure

i) A 1 ml aliquot of the eluate was transferred to a small

tube and acetate buffer of pH 7.0 (1 ml) was added; the contents were then thoroughly mixed.

ii) 0.2 ml of iodine solution was added from a 5 ml micro-burette, mixed thoroughly and then allowed to stand for exactly 1 minute.

iii) 0.2 ml of freshly prepared alkaline sulphite solution was added with mixing, and the solution was allowed to stand for exactly 1 minute.

iv) 5M acetic acid (0.2 ml) was added, with mixing, and the tube was then placed in boiling water for 3 minutes. Rearrangement of the iodine oxidation products of the catecholamine to the corresponding trihydroxyindoles in alkaline solution is a photocatalysed reaction requiring 60 minutes; however, the time period is considerably reduced by boiling at 100°C for 3 minutes (Chang, 1964). The fluorophores produced have been found to be stable for at least one hour. It should be noted that if potassium ferricyanide is used as the oxidizing agent instead of iodine, then the fluorophores produced are unstable and have to be read within 5 minutes (Anton and Sayre, 1962).

v) Blanks, for the determination of background fluorescence, were prepared in a similar way to the sample except that the alkaline sulphite solution was added before the iodine solution.

vi) Internal standards (known concentrations of noradrenaline) were included to check recoveries and quenching.

vii) Controls (standard solutions of noradrenaline) were also analysed and these values were then used in the calculation of tissue noradrenaline.

viii) The Aminco-Bowman Spectrophotofluorometer (SPF) was used to measure fluorescence.

b) Calculation of noradrenaline:

$$\text{Noradrenaline (gram/gram tissue)} = \frac{A - B}{B} \times C \times \frac{5}{1} \times \frac{1}{D} \times \frac{3}{1}$$

where: A – fluorescence of noradrenaline in the aliquot of acid eluate, minus blank, at pH 7.0

B – fluorescence of known concentration of noradrenaline (equivalent to internal standard), minus blank, at pH 7.0.

C – concentration (in gram/ml) of noradrenaline which gives fluorescence B.

D – weight of tissue

The tissue was homogenized in 5 ml of 0.4M perchloric acid and only 1 ml was used for analysis; therefore, it is necessary to multiply the results by a factor of five in order to obtain noradrenaline concentration on a gram/gram tissue basis. The relative fluorescence produced by noradrenaline was found to be linear within the concentration range used in this study.

c) Differential measurement of adrenaline and noradrenaline:

Some experiments were performed in order to measure both adrenaline and noradrenaline. This was possible because although both noradrenaline and adrenaline are converted into fluorophores at pH 7.0, only adrenaline is converted at pH 3.0. It is therefore possible to measure noradrenaline and adrenaline by taking two aliquots from one sample and following the above procedure using acetate buffers at the

two different pH's. Thus:

$$\text{Adrenaline (gram/gram tissue)} = \frac{Y - E_1}{E_1} \times C_1 \times 5 \times \frac{1}{D} \times \frac{3}{1}$$

where: C_1 - concentration (in gram/ml) of adrenaline which gives fluorescence E_1

E_1 - fluorescence of a known concentration of adrenaline (equivalent to internal standard), minus blank, at pH 3.0.

Y - fluorescence of the acid eluate, minus blank, at pH 3.0.

Since noradrenaline produces no appreciable fluorescence at pH 3.0, Y will equal the fluorescence due to the internal standard (E_1) and the amount of adrenaline in the tissue. Thus:

Noradrenaline (gram/gram tissue):

$$X - \frac{\left([(Y-E_1)E/E_1] + B\right) C \times \frac{5}{1} \times \frac{1}{D}}{B} \times \frac{3}{1}$$

where: X - fluorescence of the acid eluate, minus blank, at pH 7.0.

E - fluorescence of a known concentration of adrenaline (equivalent to internal standard), minus blank, at pH 7.0.

The ratio of adrenaline fluorescence at pH 7.0 to that at pH 3.0 is:

$$\frac{E}{E_1}$$

Thus the fluorescence due to tissue adrenaline at pH 7.0 is:

$$(Y-E_1)E/E_1$$

and the fluorescence due to noradrenaline at pH 7.0 is:

$$X - [(Y-E_1)E/E_1]$$

d) Determination of percentage (%) recovery

Internal standards (known amounts of noradrenaline, or adrenaline) were added to tissue samples prior to homogenization and the fluorescence obtained, after correction for tissue catecholamine, was compared to that obtained with a known amount of noradrenaline, or adrenaline, which was subjected only to the formation of the trihydroxyindole derivative. Thus a % yield could be obtained for each experiment and the noradrenaline and adrenaline levels were corrected accordingly.

e) Effect of normetanephrine, metaraminol, cocaine, phenoxybenzamine, ouabain and parnate on the trihydroxyindole procedure

Normetanephrine and metanephrine are not absorbed by alumina at pH 8.2 - 8.4 and thus do not affect the method of noradrenaline analysis described here (Anton and Sayre, 1962; Schneider and Gillis, 1965; Anton and Sayre, 1966).

Metaraminol forms no detectable fluorophor at either pH 3.0 or 7.0 and thus does not influence noradrenaline analysis. The structure-reactivity-relationships for the THI procedure have been well documented (see Laverty and Taylor, 1968).

Cocaine, phenoxybenzamine, parnate and ouabain were also found

to form no interfering fluorescent compounds when subjected to the THI procedure.

D. NORADRENALINE UPTAKE EXPERIMENTS

Isolated umbilical arteries of approximately 8" long were cut longitudinally so as to expose both extra- and intraluminal surfaces. Each strip was suspended in the glass apparatus illustrated in Figure 4 and was perfused with Krebs solution, mofified with 2% PVP and EDTA (5×10^{-5}M) and containing various concentrations of noradrenaline. The perfusion rate was 10 ml/min. At appropriate times, samples of the tissue were removed, washed in noradrenaline-free Krebs for one minute and analysed by the procedure previously described. The effects of phenoxybenzamine, normetanephrine, metaraminol, cocaine and ouabain on the uptake of noradrenaline by the arterial tissue were determined by perfusing the artery at a rate of 10 ml/min for 30 minutes with Krebs containing the drug, and then for a further 30 minutes with Krebs solution containing both the drug and noradrenaline. In order to determine the effect of temperature, the Krebs solution was maintained either at 0°C, 20°C or 37°C and the effects of these changes on noradrenaline accumulation was determined.

E. FLUORESCENCE MICROSCOPY

The distribution of noradrenaline in the placental tissues was studied using the formaldehyde fluorescence technique. Eranko, in 1952, observed that, after the exposure of sections of the adrenal medulla

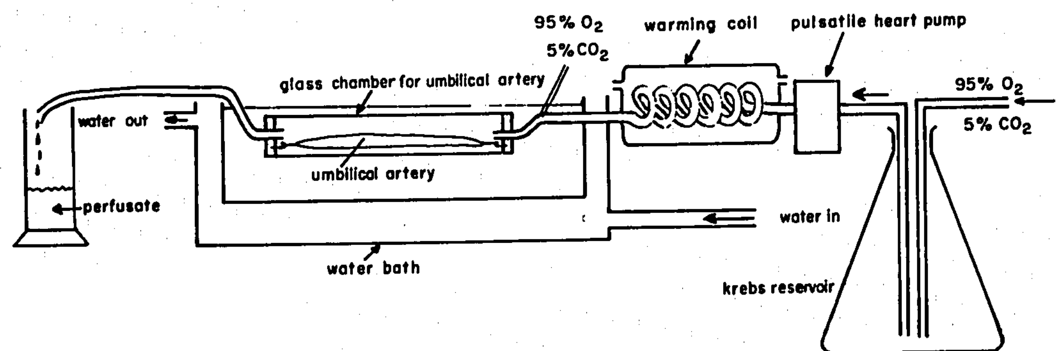


Figure 4. Apparatus for determining effect of drugs on noradrenaline uptake by umbilical arteries.

to formaldehyde solution, a fluorescence developed in some cells. With specific chemical methods these cells were then shown to contain noradrenaline. This principle was later adopted by Falck and coworkers (Falck, 1962; Falck et al., 1962; Falck and Owman, 1965) to study the distribution of catecholamines in nerve endings. Freeze-dried tissues exposed to formaldehyde vapour developed an intense fluorescence. This technique is somewhat slow because of the need to freeze-dry the tissue, embed it in paraffin wax, and then section the embedded tissues; however, a faster cryostat technique has been suggested by Ehinger et al. (1969) and an adaptation of this technique was used for this study and is described below.

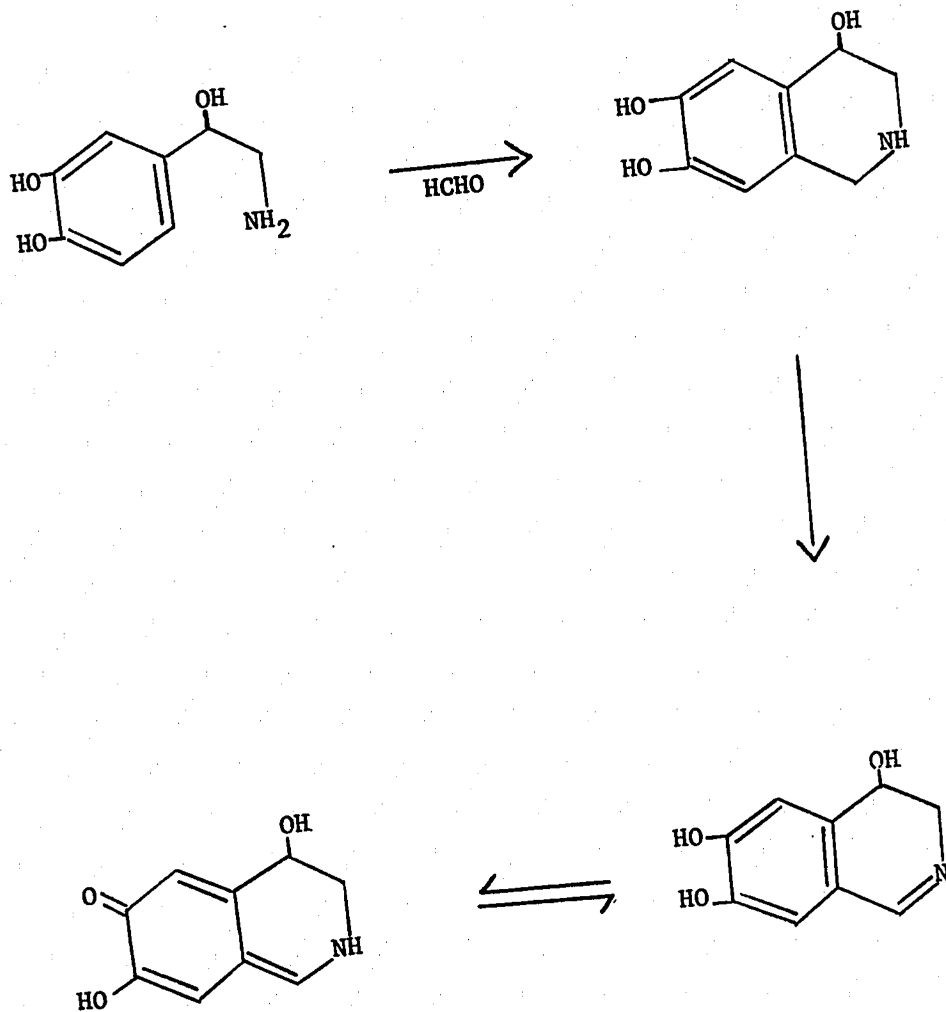
Tissues were frozen in dry ice-acetone mixture in a thermos as soon as possible after delivery, or after perfusion with noradrenaline; this mixture theoretically produces a temperature of -87°C since rapid freezing to below -30°C to -50°C prevents formation of intracellular ice crystals. The tissues were frozen using the quick freeze bar. Sections were cut to a thickness of between 4 and 10 μ using an I.E.C. razor holder and blade with anti-roll plate to prevent curling of the cut section. Sections were mounted on slides and maintained in the cryostat until sectioning was complete. They were then removed and dried for 10 minutes in a dry-air box where the air was drawn over silica gel prior to entering since the monoamine formaldehyde reaction requires the presence of dry protein. The sections were then treated at 80°C with formaldehyde gas generated from paraformaldehyde which has been equilibrated for 5 days in an atmosphere of relative humidity 15%. The sections were then mounted with cover slips on paraffin oil

and examined with the fluorescence microscope (Leitz). Photographs of various pieces of tissues were taken either using Tri-X film ASA 400 (Black and White), High Speed Ektachrome ASA 160 (Colour), Ektachrome ASA 80 (Colour), Anscochrome ASA 64 (Colour), or High Speed Anscochrome ASA 500 (Colour). It is the formation of intensely fluorescent 6,7-dihydroxy-3,4-dihydro-isoquinolines by catecholamines that enables these monoamines to be demonstrated at the cellular level (see Figure 5). The activation peak is at 410 mμ, while the fluorescence spectrum is observed at 480 mμ. The yellow 5-hydroxytryptamine fluorescence arising from 6-hydroxy-3,4-dihydro-b-carboline formation has a peak at 525 mμ and can thus be distinguished from the green fluorescence due to catecholamines. The primary amine, noradrenaline, can be distinguished from the secondary amine, adrenaline, because the adrenaline fluorescence caused by exposure to formaldehyde gas takes more than 1 hour to develop.

It was necessary to distinguish between specific catecholamine fluorescence and autofluorescence in tissues. Many tissues contain structures which emit a brown to yellow or green autofluorescence. This makes direct differentiation difficult in some situations. Two methods were used in order to distinguish specific fluorescence from autofluorescence:

1. Specimens treated with heat but without formaldehyde show only autofluorescence. By comparing sequentially-cut heat-treated sections with formaldehyde-treated sections, autofluorescence can be distinguished from catecholamine fluorescence.

2. Treatment of the formaldehyde treated tissues with water for 2-4 minutes causes the monoamine fluorophores to disappear, while



Excitation at 410 mμ
Emission at 480 mμ

Figure 5. The histochemical reaction between noradrenaline and formaldehyde.

the autofluorescence remains unaffected. The loss of catecholamine fluorescence following water treatment results in an increase in the exposure time required by the Orthomat automatic camera and this, combined with the visually obvious changes produced, enables the investigator to distinguish between autofluorescence and catecholamine fluorescence.

The study of extraneuronal noradrenaline uptake in the human umbilical artery was performed by the use of similar techniques as those employed by Avakian and Gillespie (1967) in their investigation of extraneuronal uptake of noradrenaline in the innervated central artery of the rabbit ear. Tissue sections were taken from both normal tissues and tissues incubated with noradrenaline. Krebs containing noradrenaline (10-40 μg/ml) was perfused through one umbilical artery of an isolated umbilical cord for 30 minutes and the artery was re-perfused with noradrenaline-free Krebs for 1 minute.

Perfusion with Krebs solution containing parnate, cocaine, phenoxybenzamine or normetanephrine was sometimes used to pretreat the umbilical artery for 30 minutes prior to perfusion with Krebs containing both noradrenaline and the required drug for 30 minutes. Cold Krebs at 0-4°C was also used for certain experiments; in these cases the tissue was pretreated with the cold Krebs for 30 minutes prior to perfusing with noradrenaline containing cold Krebs for a further 30 minutes. This treatment was then followed by a 1 minute wash-out period. Tissue sections were then taken and treated as described. Control studies were performed in order to demonstrate that parnate, cocaine, phenoxybenzamine and normetanephrine produced

no interference with this histochemical technique.

An approximate indication of the level of fluorescence can be obtained by using the reciprocal of the exposure time obtained by use of the Leitz Orthomat automatic camera.

F. STATISTICS

Results are given as the mean value ± standard error of the mean:

Standard error of the mean:

$$S.E._{\bar{x}} = \sqrt{\frac{\xi(x-\bar{x})^2}{n(n-1)}}$$

The students paired t-test was used, where possible, to calculate the significance of the difference between the two values which were being compared. A probability value (p) of 5% or less (<0.05) was selected as indicating that a significant difference existed between two sets of results.

$$S.E.\ diff. = \sqrt{\frac{\xi(x_{diff} - \bar{x}_{diff})^2}{n(n-1)}}$$

$$t = \frac{\bar{x}_{diff}}{S.E.\ diff}$$

III. RESULTS

III. RESULTS

A. WHOLE ORGAN PERFUSION EXPERIMENTS

1. Responses to Tyramine

Prior to testing the effects of drugs on the preparation, at least one control administration of 0.5 ml of 0.9% saline was performed to determine whether the drug solvent had any effect on the perfusion pressure. It was found that the effect of saline was negligible, causing a transient increase in perfusion pressure that was never greater than 1 mm Hg.

It was found that 17 out of 32, or approximately 50%, of the preparations tested responded to the initial administration of 5 mg tyramine when there had been no prior administration of noradrenaline. Subsequent doses of tyramine, at the same concentration, resulted in a significant ($p<0.05$) decrease in response and thus the "classical tachyphylactic" response to tyramine existed in this organ (Figure 6).

The response to tyramine, compared to the previous response, was significantly increased ($p<0.05$) and partially restored by the administration of noradrenaline (Table I) and thus the tyramine response may be at least partially dependent on tissue noradrenaline. Table I illustrates the results of nine experiments in which the response to tyramine was recorded four times in succession in order to demonstrate tachyphylaxis. Further evidence for the role of tissue noradrenaline is provided by the fact that a further 5 preparations, which were initially unresponsive to tyramine, gave contractions to tyramine

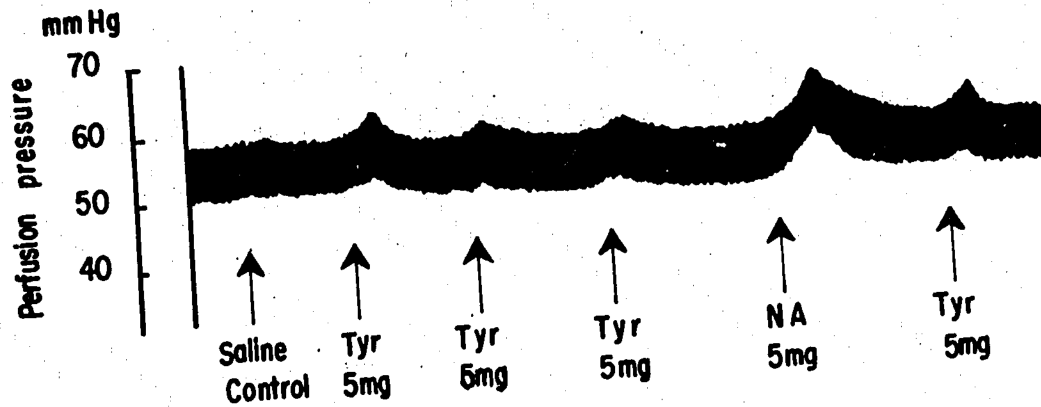


Figure 6. Noradrenaline (NA)-dependence of the response to tyramine (Tyr) in the perfused whole placenta preparation.

TABLE I

Responses to Tyramine in Perfused Placenta Preparations*

Sequence of Drug Administration†	Effect on Perfusion Pressure (mm Hg)
5 mg tyramine	4.8 ± 1.1
5 mg tyramine	3.4 ± 0.71
5 mg tyramine	2.3 ± 0.68
5 mg tyramine	2.0 ± 0.58
5 mg noradrenaline	8.0 ± 2.88
5 mg tyramine	3.2 ± 0.66

*Number of experiments = 9

†Drugs were administered to each preparation in the order described. This also applies to Tables II and III.

after exposure to noradrenaline (Table II).

2. Effect of Cocaine

Administration of cocaine alone had little or no effect on perfusion pressure (Table III and Figure 7). A significant ($p<0.05$ for paired "t" test) potentiation of the noradrenaline response by cocaine was noted (Table III). However, the degree of potentiation seen was quite small. The results illustrated by Table II also demonstrate that cocaine had little or no effect on tyramine responses, although the tachyphylactic nature of the tyramine response complicates the interpretation of these experiments.

B. NORADRENALINE ANALYSIS OF PLACENTA AND UMBILICAL CORD

Using the THI technique described in the METHODS, various standard solutions of noradrenaline were analysed and it was found that the fluorescence obtained was linear for dilutions down to the level of 1 nanogram per ml; procedure for work curves is presented in the Handbook on Aminco-Bowman Spectrophotofluorometer. The limit of sensitivity for the analysis of control amounts of noradrenaline was 1 nanogram/ml noradrenaline and since approximately $\frac{1}{2}$ gram of tissue was homogenized in 5 ml perchloric acid, the limit of sensitivity for tissue analysis is approximately 10 nanogram/gram tissue.

Tissues from various parts of fresh placenta and umbilical cord, obtained less than $\frac{1}{2}$ hour after delivery, were analysed for noradrenaline and adrenaline. The results are illustrated in Table IV. No adrenaline was detected in any of the samples and only low levels of

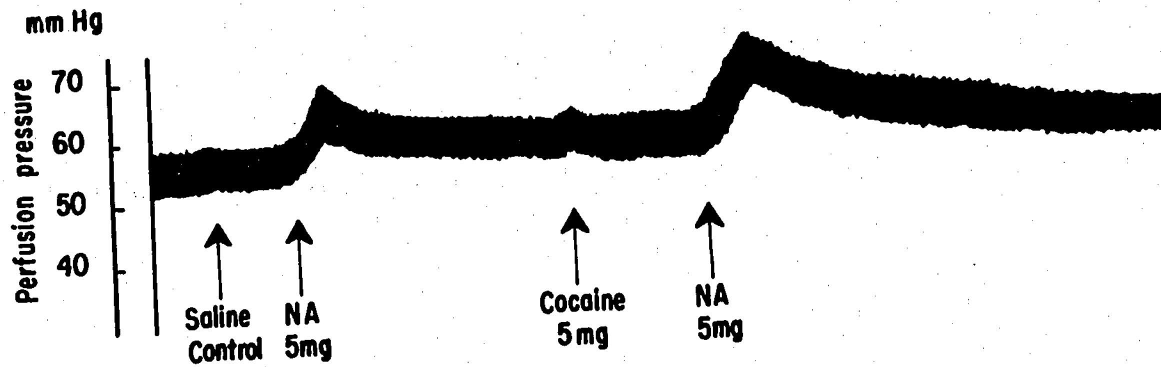


Figure 7. Effect of cocaine on the response to noradrenaline (NA) in the perfused whole placenta preparation.

TABLE II

Noradrenaline induced Tyramine Response

Sequence of Drug Administration	Number of Experiments	Effect on Perfusion Pressure (mm Hg)
5 mg tyramine	5	0.0 ± 0
5 mg tyramine	5	0.0 ± 0
5 mg noradrenaline	5	8.2 ± 1.6
5 mg tyramine	5	3.8 ± 0.85
5 mg tyramine	5	2.0 ± 0.55
5 mg cocaine	3	1.0 ± 0.00
5 mg tyramine	3	1.7 ± 0.34

TABLE III

Effect of Cocaine on the Response to Noradrenaline

in the Isolated Perfused Placenta*

Sequence of Drug Administration	Effect on Perfusion Pressure (mm Hg)
5 mg NA	8.3 ± 3.1
5 mg cocaine	1.3 ± 0.33
5 mg NA	10.0 ± 2.9†

* Number of experiments = 12

† $p<0.05$

TABLE IV

Noradrenaline Content of Human Umbilical Cord and Placenta

(Determined by the trihydroxyindole method)

Region	Number of samples	Mean NA content (µg/g)
Placental villi	8	0.045 ± 0.003
Umbilical cord (whole tissue)	12	0.075 ± 0.002
Umbilical cord arteries	12	0.093 ± 0.023
Umbilical cord veins	4	0.090 ± 0.010

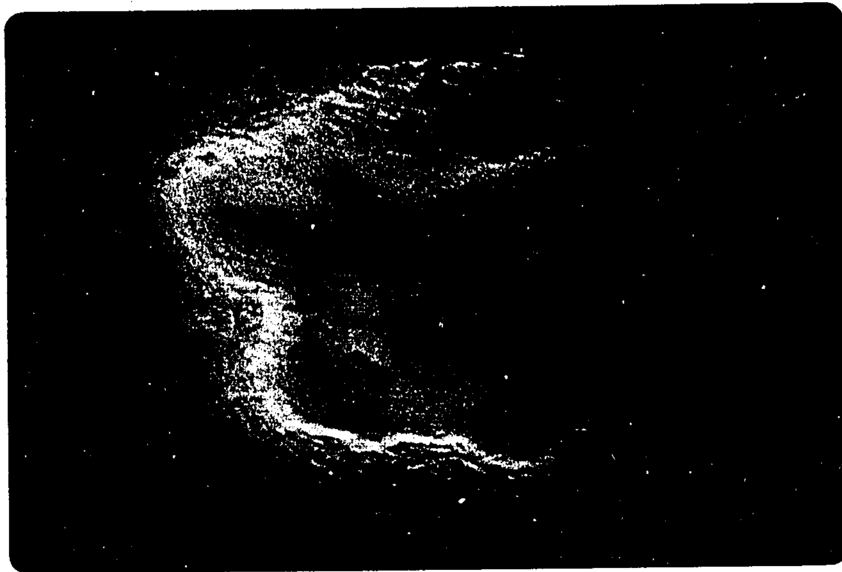
noradrenaline were found.

C. FLUORESCENCE MICROSCOPY OF UMBILICAL CORD TISSUE

1. Normal Tissues

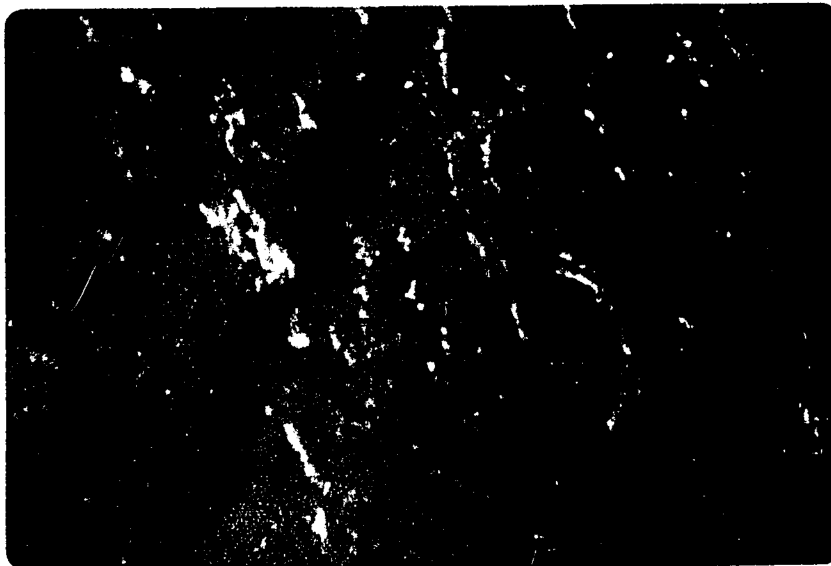
Using the Falck technique (*cf.* METHODS), sections from normal, fresh umbilical cord were examined for adrenergic nerve endings. Sections from 24 specimens have been examined. Six sequential sections from two or three areas of the umbilical cord were taken; five were treated with paraformaldehyde at 80°C for one hour and the remaining section subjected to heat only at 80°C for one hour. The heat treated section demonstrated autofluorescence only; no noradrenaline fluorescence was observed. After microscopic examination, one or two sections from each area were treated with water in order to remove catecholamine fluorophores and thus enabled the distinction to be made between tissue autofluorescence and possible catecholamine fluorescence.

Examination of these sections failed to reveal any structures which resembled adrenergic nerve endings. Photograph 1 indicates a transverse section through an umbilical artery. An example of adrenergic nerve endings from an innervated tissue is illustrated in photograph 2 which shows a section of rat heart and photograph 3 which shows the same section after treatment with water. While it is not impossible that the period of delay between delivery and sectioning could have caused loss of noradrenaline from nerve terminals, this suggestion is most improbable. The period between delivery and sectioning was seldom longer than 30 minutes, and in other tissues

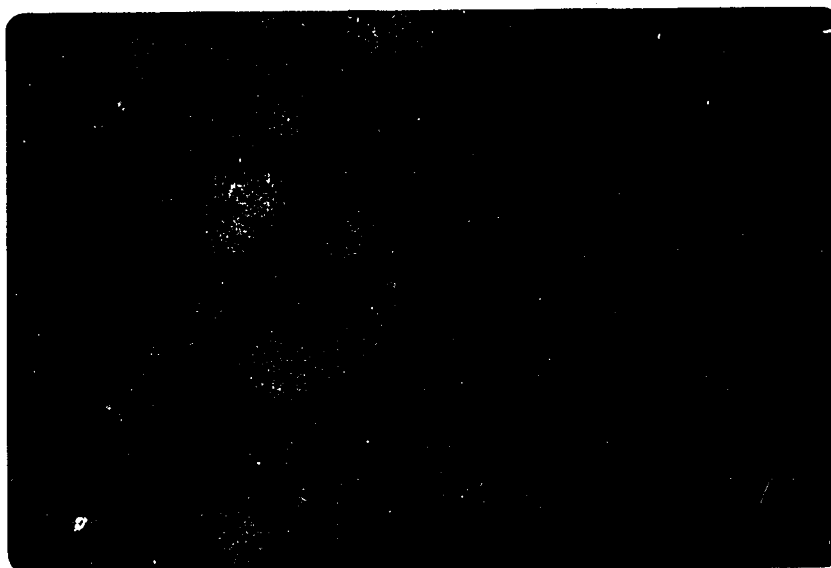


Photograph 1: Transverse Section (T.S.) of normal umbilical cord to show umbilical artery.
Photograph taken with high speed Ektachrome at ASA 250
Exposure = 2 min 8 sec. Magnification: x 20.

Photograph 2:



Photograph 3:



Photograph 2: Rat heart section to show adrenergic nerve endings. Taken with high speed Ektachrome at ASA 250. Exposure = 2 min 15 sec. Magnification: x 50

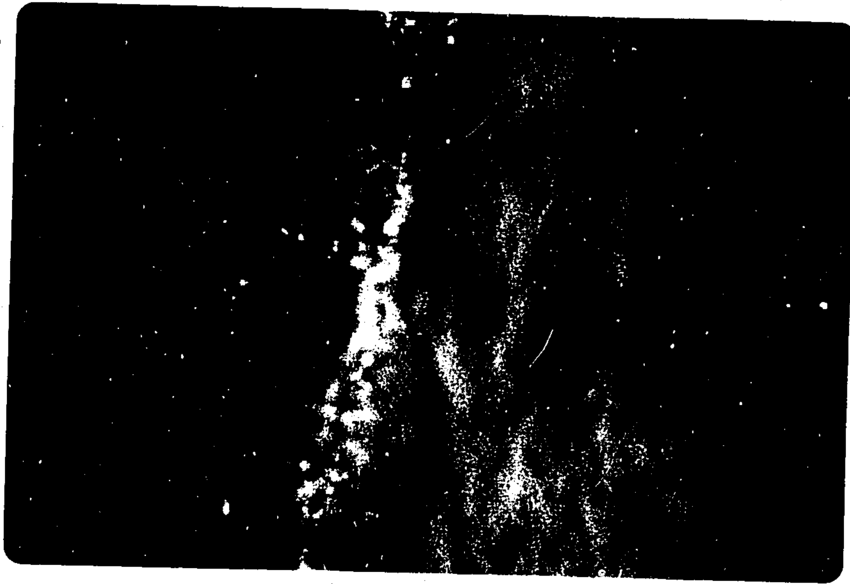
Photograph 3: As photograph 2 but after water treatment. Exposure = 2 min 30 sec.

which receive sympathetic innervation it was found that, even after one hour delay, little diffusion of transmitter had occurred. This is shown in photographs 4 and 5. Photograph 4 shows the adrenergic nerve terminals in rabbit aorta where the tissue was frozen within 5 minutes of death. Photograph 5 shows the same preparation after exposure to room temperature for one hour prior to freezing. In both preparations the adrenergic nerve terminals may be clearly seen. Photograph 6 shows the adrenergic nerve terminals of coronary blood vessels; this tissue was also exposed to room conditions for one hour prior to freezing and sectioning.

2. Incubated Tissues

Further data concerning the possibility of nerve endings in the umbilical cord has been obtained by perfusing the cord with Krebs solution containing noradrenaline and then sectioning these cords and examining for noradrenaline-induced adrenergic fluorescence. The purpose of these noradrenaline incubation experiments is to attempt to saturate with noradrenaline any adrenergic nerves which may exist in the cord and thus make them readily detectable by fluorescence microscopy. Eight experiments were performed in this series using a noradrenaline concentration of 40 μg/ml and five experiments using a noradrenaline concentration of 10 μg/ml. The results are illustrated by the representative photographs 11 to 16. Six sequential sections were taken from two parts of the incubated cord and five of these sections were then treated with paraformaldehyde at 80°C for one hour and the remaining one was heat-treated at 80°C for one hour. For each experiment, sections were also taken from an innervated tissue, usually spleen,

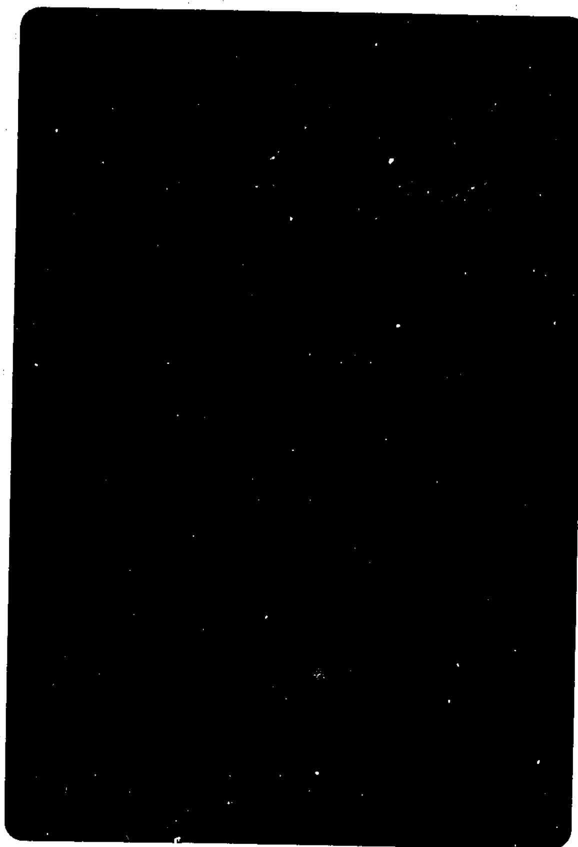
Photograph 4:



Photograph 5:

Photograph 4: Rabbit aorta to demonstrate adrenergic nerve endings in adventitia. Tissue frozen and sectioned 5 min. after death. Taken with Anscochrome ASA 64 at ASA 320. Exposure = 1 min 30 sec. Magnification: x 50.

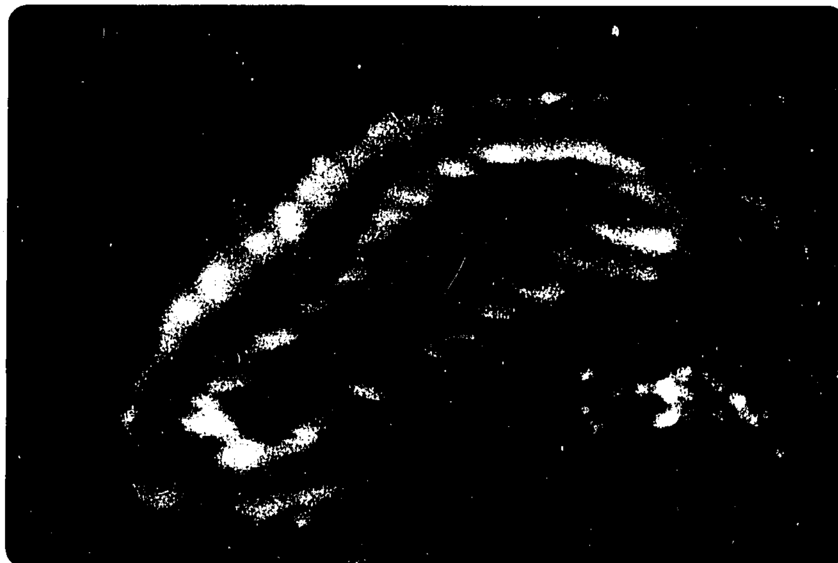
Photograph 5: Rabbit aorta as above but tissue frozen and sectioned 1 hour after death. Exposure = 1 min 30 sec.



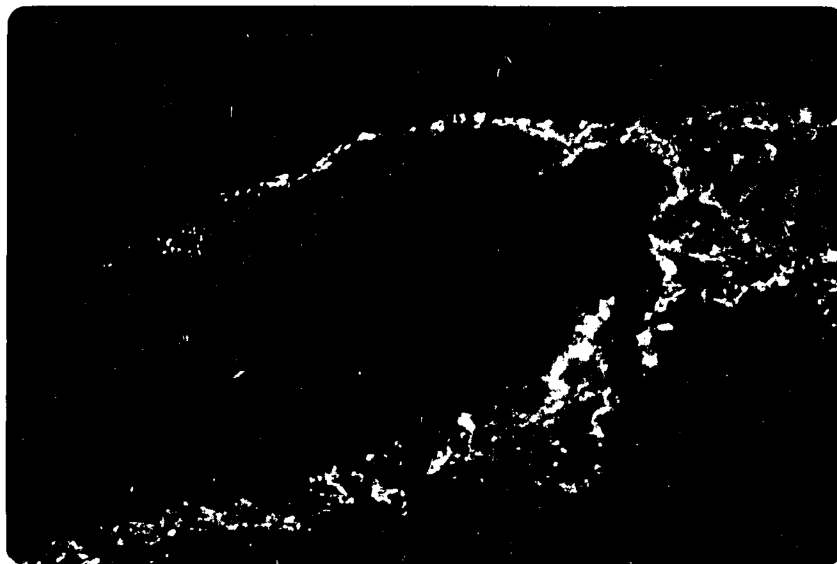
Photograph 6: Blood vessels in rabbit heart detected in tissue which was frozen and sectioned 1 hour after death of animal. Taken with Anscochrome ASA 64 at ASA 320. Exposure = 1 min. 39 sec. Magnification: x 50.

heart or thoracic aorta of rabbit, guinea-pig or rat in order to compare these results to those obtained using umbilical cord. The detection of nerve endings in innervated tissues demonstrated that the conditions of the experiments were correct and adrenergic nerve endings were detectable. Photograph 7 illustrates a section from a rabbit spleen blood vessel which was stored overnight in Krebs in order to partially deplete the noradrenaline stores. Photograph 8 represents a section from the same spleen as photograph 7 but, before sectioning, this piece of tissue was incubated in Krebs containing 10 μg/ml noradrenaline for 30 minutes. It is seen, in photograph 8, that the fluorescence of the nerve endings have been restored and the light-green fluorescence in the smooth muscle of the vessel represents extraneuronal noradrenaline uptake. Photograph 9 and 10 illustrate unilocular fat cells from the rabbit before and after incubation with noradrenaline (10 μg/ml) prior to sectioning. Photograph 11 illustrates a transverse section of an umbilical artery after noradrenaline treatment, and photograph 12 represents the same section after water treatment to remove the catecholamine fluorophores. Photograph 13 illustrates the elastic fibres from an umbilical artery and photograph 14 shows the same areas after water treatment. This series of photographs illustrates that no adrenergic nerves can be detected in these sections and fluorescent areas, which are seen in the sections, are actually autofluorescence usually due to elastic fibres. Exposure times for the photography can be used to indicate changes in catecholamine fluorescence before and after water treatment. The exposure time for photograph 11 before water treatment was 1 minute

Photograph 7:



Photograph 8:



Photograph 7: Blood vessel, with adrenergic nerve endings, in rabbit spleen after storage overnight in Krebs. Taken with Anscochrome ASA 64 at ASA 320. Exposure = 2 min. 45 sec. Magnification: x 50.

Photograph 8: Blood vessel in same rabbit spleen as above but after incubation with 10 µg/ml noradrenaline for 30 min. Taken with Anscochrome ASA 64 at ASA 320. Exposure = 55 sec. Magnification: x 50.

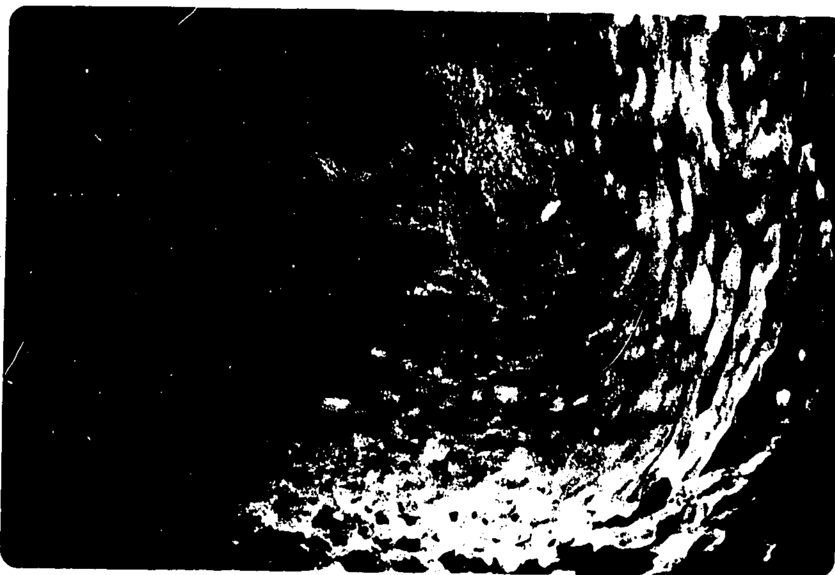
Photograph 9:

Photograph 10:

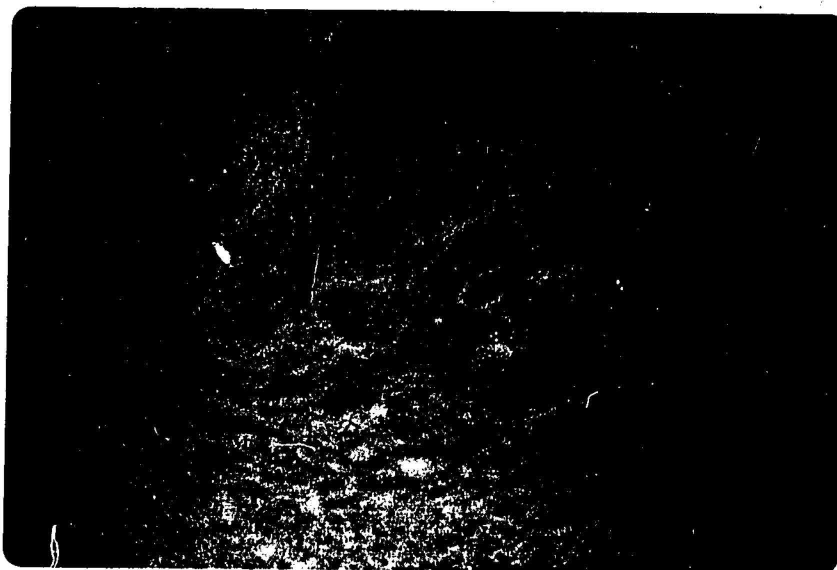
Photograph 9: Unilocular fat cells from rabbit spleen. Taken with Anscochrome ASA 64 at ASA 320. Exposure = 4 min 15 sec. Magnification: x 80.

Photograph 10: As above but after incubation with 10 μg/ml noradrenaline for 30 min. Exposure = 3 min 15 sec.

Photograph 11:



Photograph 12:

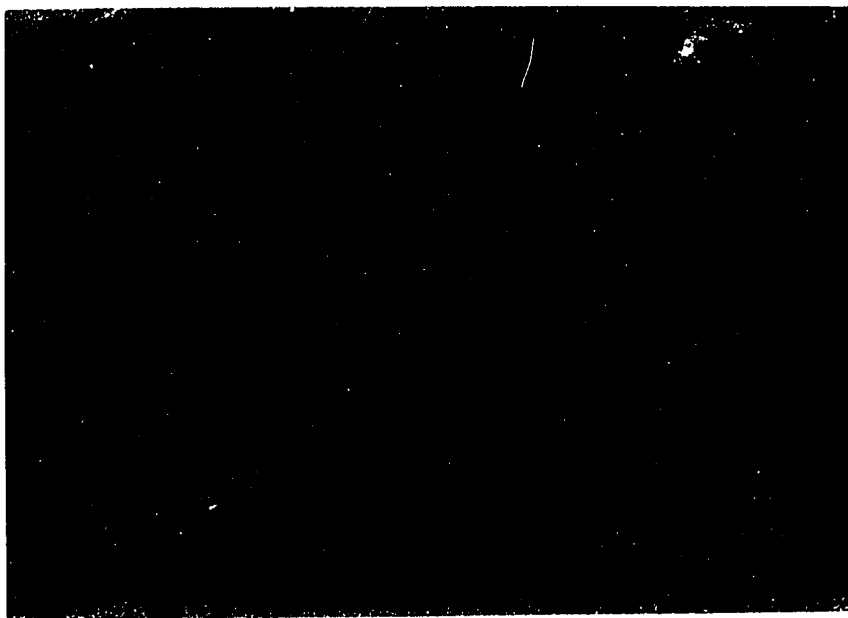


Photograph 11: Umbilical artery after incubation with 40 μg/ml noradrenaline for 30 min. Taken with Ektachrome at ASA 160. Exposure = 1 min 6 sec. Magnification: x 50.

Photograph 12: As above, but after water treatment. Exposure = 12 min.

Photograph 13:

Photograph 14:



Photograph 13: Umbilical artery elastic fibers after incubation with 40 μg/ml noradrenaline for 30 min. Taken with Tri-X film at ASA 200. Exposure = 57 sec. Magnification: x 50.

Photograph 14: As above but after water treatment. Exposure = 1 min 40 sec.

6 seconds, while after water treatment, the exposure time (photograph 12) was 12 minutes. One can use the reciprocal of these exposure times as an indication of the relative brightness of the tissue. This background fluorescence represents extraneuronal uptake of noradrenaline.

Photograph 15 illustrates another umbilical artery section after incubation with noradrenaline, whilst photograph 16 illustrates a sequential section to that of photograph 15 which was heat treated instead of exposed to paraformaldehyde. After heat treatment, only autofluorescence is observed.

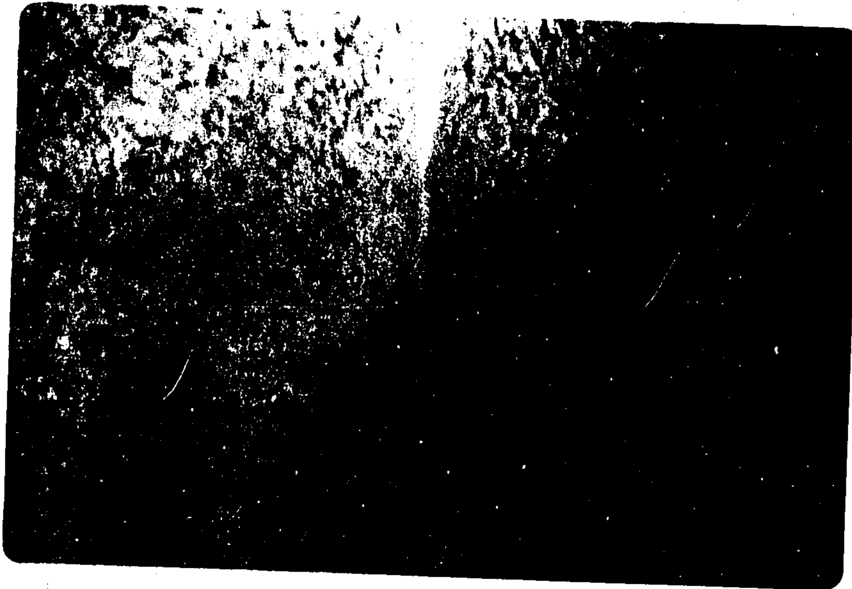
3. Effect of MAO inhibition on Noradrenaline Uptake

Photograph 17 illustrates the tissue fluorescence in a section from a cord which was pretreated for 30 minutes with the MAO inhibitor parnate (10^{-4}M) prior to incubation with noradrenaline (40 μg/ml) for 30 minutes. This is an illustration of one of a series of four experiments which were performed to determine whether inhibition of MAO in the cord could enable the visualization of adrenergic nerves in the tissue by virtue of inhibiting the metabolism of noradrenaline and thus facilitating its detection by fluorescent microscopy. As illustrated, no such nerve endings were found.

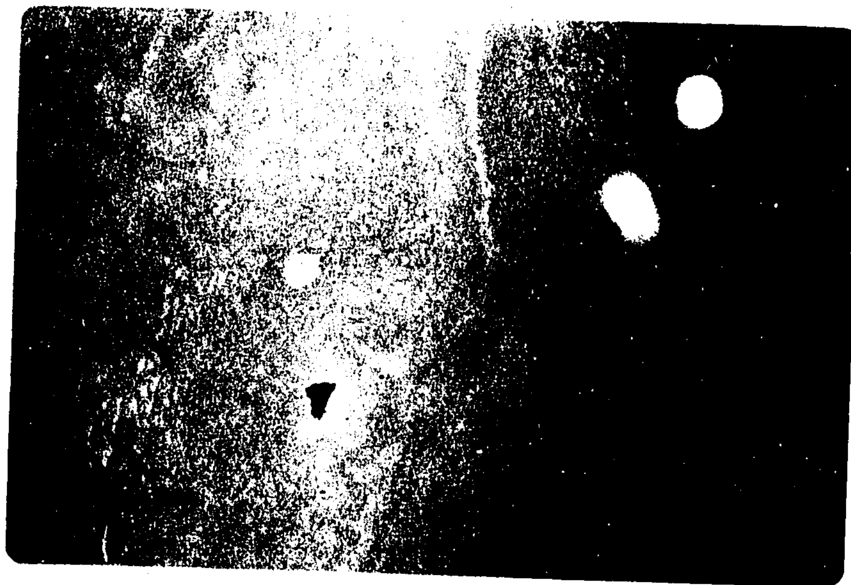
4. 5-hydroxytryptamine Accumulation in the Umbilical Cord

Four series of experiments have been performed using 5-hydroxytryptamine (10 μg/ml) and the results would seem to illustrate a similar tissue distribution to that of noradrenaline. No tryptaminergic nerves were detectable.

Photograph 15:



Photograph 16:



Photograph 15: Umbilical artery after incubation with 10 μg/ml noradrenaline for 30 min. Taken with Ektachrome at ASA 100. Exposure = 4 min 12 sec. Magnification: x 20.

Photograph 16: Sequential section to above but heat treated with no paraformaldehyde. Exposure = 7 min 25 sec.

Photograph 17: Umbilical artery pretreated with 10^{-4}M parnate for 30 min. and then incubated with 40 μg/ml noradrenaline for a further 30 min.
Taken with high speed Ektachrome at ASA 200.
Exposure = 1 min 2 sec. Magnification: x 50.

5. Distribution of Noradrenaline in the Placental Villi

Sections of placental villi from five different placenta were incubated in Krebs solution containing noradrenaline (40 μg/ml). Histochemical fluorescence studies failed to reveal any structures resembling adrenergic nerve endings.

6. Effect of Temperature on Noradrenaline Uptake in the Umbilical Cord

Since the earlier experiments have illustrated that noradrenaline can be accumulated by the umbilical cord tissue, although not into adrenergic nerve endings, a series of experiments were performed to determine whether perfusion of the tissue at 0°C and 20°C would affect the distribution of tissue noradrenaline. The concentration of noradrenaline in the perfusate was 40 μg/ml. Four experiments at 0°C and four at 20°C were performed and the fluorescence of experimental tissues were compared to those of control tissues which were perfused at a temperature of 37°C. No detectable difference in the fluorescence of the tissues was noted. Reduction of the temperature of the perfusion medium thus had little effect on the noradrenaline uptake. The increase in exposure times of control and experimental tissues was similar after water treatment, representing a minimum of a two-fold increase.

7. Effect of Phenoxybenzamine on Noradrenaline Distribution in the Umbilical Cord

Experiments were then performed to determine the effect of 1 μg/ml and 10 μg/ml phenoxybenzamine on noradrenaline accumulation from perfusate containing 40 μg/ml noradrenaline. The cord artery was

perfused with Krebs containing both noradrenaline and phenoxybenzamine and tissue sections were examined as previously described. No detectable difference in the fluorescence of the tissue was noted in the three experiments carried out at each concentration of antagonist. Phenoxybenzamine did not produce a detectable block of the noradrenaline uptake.

8. Effect of Cocaine on Noradrenaline Distribution in the Umbilical Cord

Four experiments were performed to demonstrate the effect of 1 μg/ml and 10 μg/ml cocaine on noradrenaline accumulation. The perfusate concentration of noradrenaline was 40 μg/ml. The experimental procedure was the same as that outlined above (section 7). No detectable difference in the distribution and accumulation of the noradrenaline between control and experimental specimens was noted.

9. Effect of Normetanephrine on Noradrenaline Distribution in the Umbilical Cord

Four experiments were performed to demonstrate the effect of 10 μg/ml and 50 μg/ml normetanephrine on the accumulation of noradrenaline (40 μg/ml) in the umbilical cord. The conditions were the same as those for similar experiments involving phenoxybenzamine and cocaine and no detectable difference in the distribution and accumulation of noradrenaline between control and test specimens was noted.

10. Loss of Accumulated Noradrenaline from the Tissue

After perfusion of the umbilical cord with Krebs containing

noradrenaline (40 μg/ml) for 30 minutes, the cord was re-perfused with noradrenaline free Krebs and, by taking sections of the cord at various time intervals it was found that although some tissue fluorescence was detectable after 30 minutes, all detectable fluorescence had disappeared within 45 minutes.

D. UPTAKE OF NORADRENALINE BY ISOLATED UMBILICAL ARTERIES

1. Accumulation of Noradrenaline

The isolated umbilical artery strip was perfused with Krebs containing noradrenaline (0.1 μg/ml to 40 μg/ml) for various incubation times of 1, 5, 15, 30 and 60 minutes. The tissue was then incubated in noradrenaline-free Krebs for exactly one minute and the noradrenaline content of the tissue determined. The results are illustrated in Table V.

2. Loss of Noradrenaline from Tissue

After perfusing the tissue for 15 minutes with Krebs containing noradrenaline (40 μg/ml), the tissue was re-perfused with noradrenaline free Krebs and samples of the tissue taken at 0, 1, 5, 15, 30, 45 and 60 minutes. The noradrenaline level in these samples was calculated and the results are indicated in Table VI. Initially the noradrenaline is lost very rapidly but the efflux slows down and noradrenaline is still detectable in the tissue after 60 minutes when the experiments were terminated.

TABLE V

Noradrenaline Uptake

NA μg/ml	No. Expts.	μg Noradrenaline/gram tissue					
		Time in Minutes					
		1	5	15	30	60	90
40	13	9.16±1.03	16.57±1.95	27.93±2.32	32.96±4.13	37.6 ±2.78	
10	6		2.97±1.33	7.21±1.33	8.02±2.45	8.77±2.74	14.39*
1	8		0.43±0.03		0.71±0.08		
0.1	4		0.10±0.08		0.20±0.10		

*two experiments only

TABLE VI

Noradrenaline Loss from Tissue after Previous

Incubation in 40 μg/ml Noradrenaline-Krebs

for 15 minutes*

Time in Minutes	μg Noradrenaline/gram tissue
0	42.9 ± 4.02
1	27.23 ± 1.5
5	13.0 ± 1.40
15	2.91 ± 0.65
30	0.4 ± 0.18
45	0.24 ± 0.08
60	0.075± 0.01

*Number of Experiments = 5

3. Effect of Temperature on the Uptake of Noradrenaline

The effect of temperature on noradrenaline uptake by the umbilical artery was determined by lowering the temperature of the noradrenaline containing perfusate to 0-2°C and to 18-20°C. The results are illustrated in Table VII and it was found that the noradrenaline uptake, both at 0 and 20°C, was significantly ($p<0.05$) different from control values at 37-38°C. The Q_{10} calculated from these results was 1.2.

4. Effect of Cocaine on Uptake of Noradrenaline

Strips of umbilical artery were perfused with Krebs solution containing cocaine for 30 minutes at 37°C and then re-perfused with Krebs containing both cocaine and noradrenaline for a further 30 minutes. The cocaine and noradrenaline concentrations used are illustrated in Table VIII. The tissue was perfused with normal Krebs at 37°C for 1 minute and a sample was taken and analysed for noradrenaline. It was found that cocaine at a concentration of 3.3×10^{-4}M was capable of significantly (<0.05) decreasing noradrenaline (2.4×10^{-4}M and 6×10^{-5}M) uptake. Cocaine at a concentration of 3.3×10^{-5}M to 3.3×10^{-7}M had no significant ($p<0.05$) effect on noradrenaline (6×10^{-5}M and 6×10^{-6}M) uptake.

5. Effect of Various Agents on Uptake of Noradrenaline

The effect of various concentrations of drugs on noradrenaline uptake was determined by using a similar technique to that described for the effect of cocaine on noradrenaline uptake (cf. section 4 above). The results and concentrations of drugs used are indicated in Table IX.

TABLE VII

Effect of Temperature on Noradrenaline Uptake*

°C	Noradrenaline (μg/ml)	% Inhibition
0 - 2	40	30.5 ± 3.54
0 - 2	10	20.5 ± 1.15
20	40	9.0 ± 1.65
20	10	7.5 ± 0.9

* Number of experiments = 4

TABLE VIII

Effect of Cocaine on the Uptake of Noradrenaline by the Umbilical Artery

Cocaine Concentration *		Noradrenaline Concentration *		% Inhibition of noradrenaline uptake	Significance†	n
g/ml	M	g/ml	M			
10^{-4}	3.3×10^{-4}	4×10^{-5}	2.4×10^{-4}	12.3 ± 0.95	<0.05	4
10^{-4}	3.3×10^{-4}	10^{-5}	6×10^{-5}	15.6 ± 2.14	<0.05	4
10^{-5}	3.3×10^{-5}	10^{-5}	6×10^{-5}	2.0 ± 0.7	N.S.	5
10^{-5}	3.3×10^{-5}	10^{-6}	6×10^{-6}	1.0 ± 7.5††	N.S.	5
10^{-6}	3.3×10^{-6}	10^{-5}	6×10^{-5}	0.75 ± 1.1††	N.S.	4
10^{-6}	3.3×10^{-6}	10^{-6}	6×10^{-6}	1.25 ± 2.5††	N.S.	5
10^{-7}	3.3×10^{-7}	10^{-5}	6×10^{-5}	0.25 ± 1.1††	N.S.	4
10^{-7}	3.3×10^{-7}	10^{-6}	6×10^{-6}	1.5 ± 2.3††	N.S.	5

* To avoid confusion in comparing these results with those of other workers, drugs concentrations in this and subsequent tables are expressed both as g/ml and molarity.

† paired "t" test comparison to control, as in all subsequent tables (unless otherwise stated).

†† enhancement of uptake noted in some experiments.

a) Phenoxybenzamine

It was found that phenoxybenzamine at a concentration of 3.7×10^{-4}M or 3.7×10^{-5}M could significantly ($p<0.05$) decrease noradrenaline uptake. Phenoxybenzamine at a concentration of 3.7×10^{-6}M had no significant effect on noradrenaline uptake ($p>0.05$).

b) Ouabain pretreatment

Both 1.7×10^{-4}M and 1.7×10^{-5}M ouabain were found to cause a significant ($p<0.05$) decrease in the uptake of noradrenaline (Table IX).

c) Normetanephrine

The results illustrate that both 2.2×10^{-4}M and 5.4×10^{-3}M normetanephrine can significantly ($p<0.05$) decrease the uptake of noradrenaline (at 2.4×10^{-4}M and 6×10^{-5}M) from the medium.

d) Metaraminol

The results demonstrate that metaraminol, at 6×10^{-5}M does not significantly ($p>0.05$) affect the uptake of 2.4×10^{-4}M or 6×10^{-5}M noradrenaline.

e) Parnate (MAO inhibitor) pretreatment

The results illustrate that parnate pretreatment of the tissue has no significant effect on the uptake of noradrenaline ($p>0.05$).

E. ISOLATED TISSUE EXPERIMENTS

1. General Pharmacology

The approximate ED_{50}'s for various agonists which were used

TABLE IX

Effect of Various Agents on the Uptake of Noradrenaline by the Umbilical Artery*

Drug	Drug Concentration g/ml	Drug Concentration M	Noradrenaline Concentration g/ml	Noradrenaline Concentration M	% Inhibition of Uptake	p
POB	10^{-4}	3.7×10^{-4}	4×10^{-5}	2.4×10^{-4}	29.6 ±9.77	<0.05
POB	10^{-5}	3.7×10^{-5}	4×10^{-5}	2.4×10^{-4}	5.5 ±0.9	<0.05
POB	10^{-5}	3.7×10^{-5}	10^{-5}	6×10^{-5}	8.0 ±1.1	<0.05
POB	10^{-6}	3.7×10^{-6}	10^{-5}	6×10^{-5}	0.5 ±1.3	N.S.
Ouabain	10^{-4}	1.7×10^{-4}	10^{-5}	6×10^{-5}	32.0 ±1.27	<0.05
Ouabain	10^{-5}	1.7×10^{-5}	10^{-5}	6×10^{-5}	8.25±0.9	<0.05
NMN	5×10^{-5}	2.2×10^{-4}	4×10^{-5}	2.4×10^{-4}	18.25±1.7	<0.05
NMN	10^{-5}	5.4×10^{-5}	4×10^{-5}	2.4×10^{-4}	5.25±1.0	<0.05
NMN	10^{-5}	5.4×10^{-5}	10^{-5}	6×10^{-5}	9.0 ±1.3	<0.05
Metaraminol	10^{-5}	6×10^{-5}	4×10^{-5}	2.4×10^{-4}	0.8 ±1.1†	N.S.
Metaraminol	10^{-5}	6×10^{-5}	10^{-5}	6×10^{-5}	0.25±0.9†	N.S.
Parnate	1.3×10^{-5}	10^{-4}	10^{-6}	6×10^{-6}	4.1 ±3.72†	N.S.

* Number of experiments =4

† Enhancement of uptake noted in some experiments.

in this investigation are indicated in Table X. Most helical umbilical artery strips responded to 5-hydroxytryptamine, histamine and potassium chloride, whereas responses of the preparation to noradrenaline could not always be demonstrated. A response to tyramine in the preparation could be produced by one of two methods:

a) occasionally by initial administration of tyramine to the preparation, and also prior exposure of the tissue to noradrenaline whereupon the response to tyramine showed rapid tachyphylaxis, or

b) prolonged exposure of the tissue to tyramine for 2 to $2\frac{1}{2}$ hours, with repeated administration of tyramine followed by washout and readministration every 10-15 minutes, resulted in a tissue contracture. This response increased in size upon continued exposure to tyramine, "reverse tachyphylaxis", for 2 to 3 doses and a standard response was then obtained which remained fairly constant for several hours (Figure 8). This tyramine response will henceforth be referred to as an induced tyramine response.

As illustrated in Table X, the tissue was most sensitive to 5-hydroxytryptamine. Angiotensin, synthetic oxytocin and acetylcholine were also administered to some preparations, and it was found that responses to angiotensin (9.7×10^{-7} to 9.7×10^{-6}M), synthetic oxytocin (1 - 20 mU) and acetylcholine (3.5×10^{-5} to 3.5×10^{-6}M) underwent rapid tachyphylaxis (Figure 9). Isopropylnoradrenaline, a β-receptor agonist (4.7×10^{-7} to 4.7×10^{-5}M),was found to have no effect either on a normal muscle strip or on a drug contracted tissue. Phentolamine was used to block the α-receptors and then

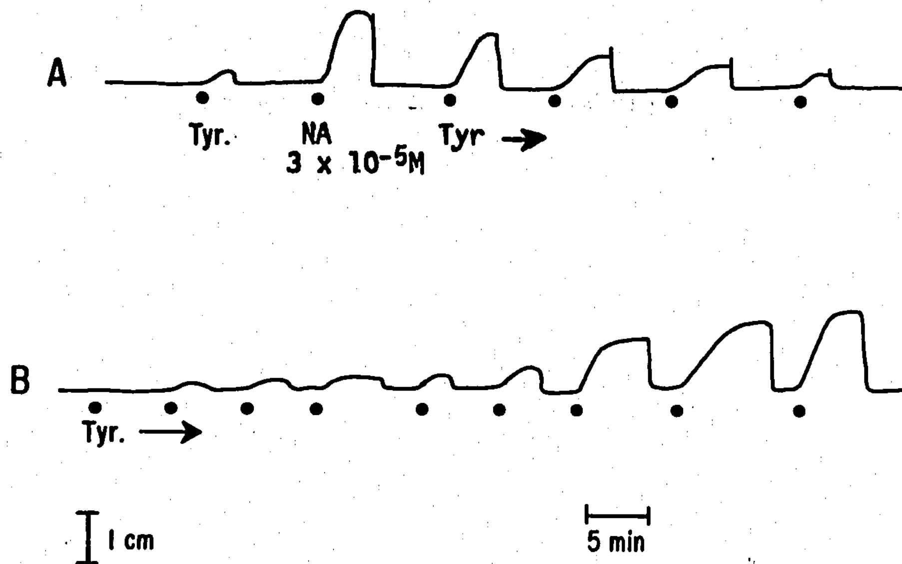


Figure 8. Responses to tyramine in the isolated umbilical artery.

A: Tachyphylactic nature of the response to 3.65 x 10^{-4}M tyramine (Tyr) in the umbilical artery.

B: Induced-tyramine response produced by continued re-exposure to 3.65 x 10^{-4}M tyramine in the umbilical artery.

• indicates drug administration and applies to all subsequent figures of this nature.

TABLE X

Approximate ED_{50}'s for Agonists

Agonist	ED_{50} g/ml	ED_{50} M	Concentration Range g/ml	Concentration Range M
5-HT	2×10^{-8}	1.26×10^{-7}	5×10^{-9} to 5×10^{-7}	3.15×10^{-8} to 3.15×10^{-6}
Histamine	2×10^{-7}	1.8×10^{-6}	5×10^{-8} to 10^{-6}	4.5×10^{-7} to 9×10^{-6}
Noradrenaline	5×10^{-6}	3×10^{-5}	10^{-6} to 10^{-5}	3×10^{-6} to 6×10^{-5}
Tyramine	5×10^{-5}	3.65×10^{-4}	10^{-5} to 10^{-4}	7.3×10^{-6} to 7.3×10^{-7}
KCl		4×10^{-2}		1.5×10^{-2} to 6×10^{-2}

isopropylnoradrenaline was re-tested in these preparations; however, no β-adrenergic-mediated relaxation could be detected. It is thus unlikely that the β-adrenergic relaxation was masked by a contracture effect of isopropylnoradrenaline on α-receptors.

2. Effect of Cocaine on Agonist Response

Tables XI to XV and Figures 10 to 14 illustrate the effects of various concentration of cocaine on responses to noradrenaline, induced-tyramine, 5-hydroxytryptamine, histamine and potassium chloride ED_{50}'s. Standard, reproducible responses were first obtained, and then the effect of cocaine was determined by pretreating the tissue with cocaine for 5 minutes and then adding the agonist. Maximum total exposure time to the drugs was 13 minutes. Significance was determined by comparing the prior control response, where no cocaine was used, to the response obtained in the presence of cocaine using the paired "t" test. In all cases, it was found that cocaine, by itself, had no excitatory effect on the tissue within the same exposure time which had a maximum of 13 minutes. A similar quantity of 0.9% saline (0.2 ml) was also found to have no significant effect on the responses to noradrenaline.

The response to noradrenaline in the presence of 1.7×10^{-7}M to 3.3×10^{-5}M cocaine was found to be significantly increased (Table XI). The response to tyramine in the presence of 1.7×10^{-6}M to 1.7×10^{-5}M cocaine was also significantly increased (Table XII). The same range of cocaine concentrations was capable of potentiating both the responses to noradrenaline and tyramine. The response to 5-hydroxytryptamine

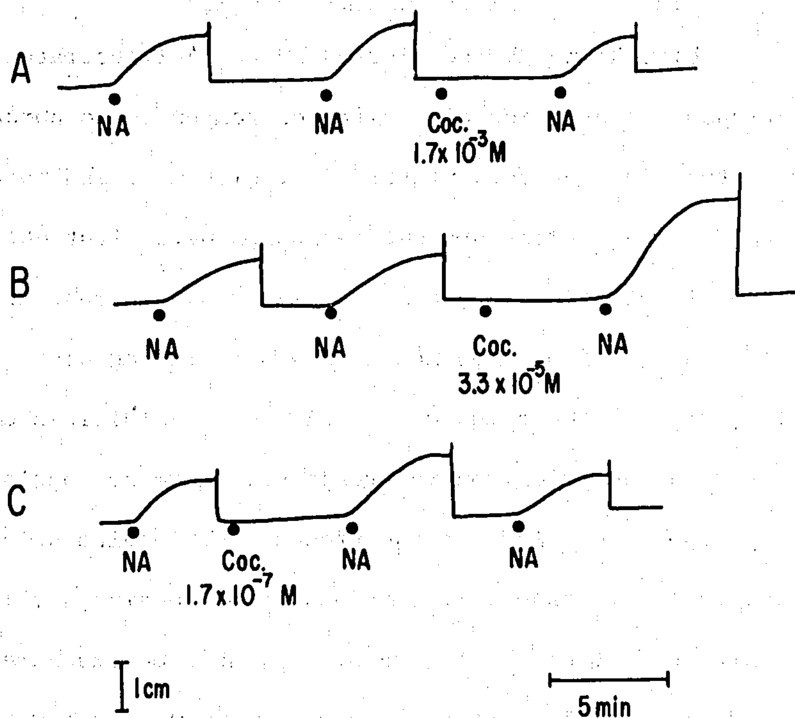


Figure 10. Effect of cocaine on the responses to noradrenaline in the isolated umbilical artery.

A: Effect of 1.7 x 10^{-3}M cocaine (Coc) on the response to 3 x 10^{-5}M noradrenaline (NA)

B: Effect of 3.3 x 10^{-5}M cocaine on the response to 3 x 10^{-5}M noradrenaline.

C: Effect of 1.7 x 10^{-7}M cocaine on the response to 3 x 10^{-5}M noradrenaline.

TABLE XI

Effect of Cocaine on Noradrenaline

ED_{50} ($3 \times 10^{-5}M$)

Cocaine g/ml	Cocaine M	Mean Effect %↑ or %↓ ±S.E.	No. Expts.	p
5×10^{-8}	1.7×10^{-7}	+39 ± 19.0	5	<0.05
5×10^{-7}	1.7×10^{-6}	+115 ± 40.9	13	<0.05
5×10^{-6}	1.7×10^{-5}	+142 ± 8.6	6	<0.05
10^{-5}	3.3×10^{-5}	+174 ± 38.3	14	<0.05
5×10^{-5}	1.7×10^{-4}	+31.3± 20.8	13	N.S.
5×10^{-4}	1.7×10^{-3}	-64 ± 15.6	6	<0.05

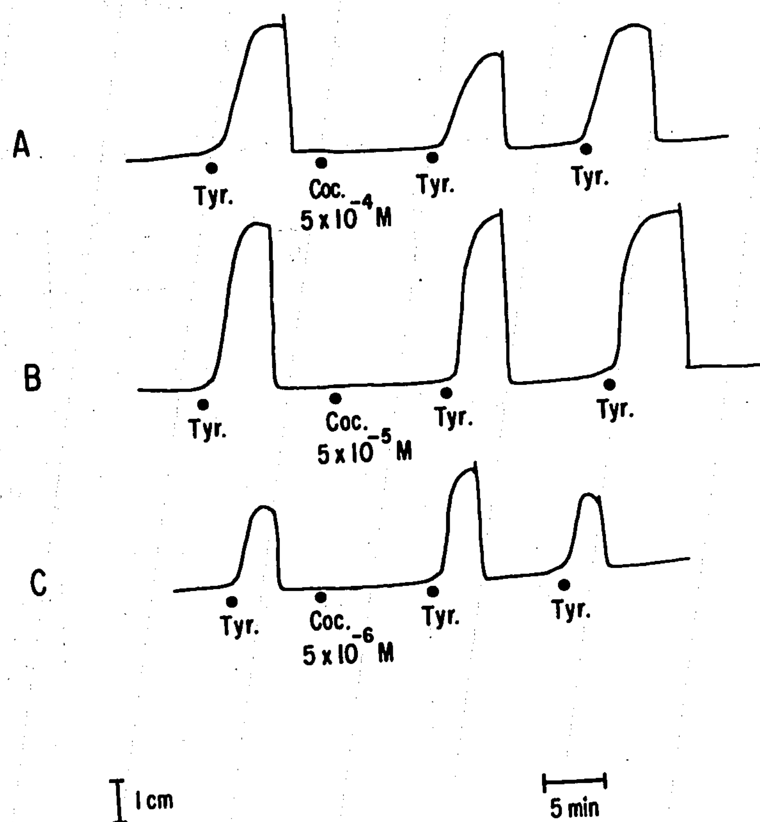


Figure 11. Effect of cocaine on the induced tyramine response in the isolated umbilical artery.

A: Effect of 5×10^{-4}M cocaine (Coc) on the response to 3.65×10^{-4}M tyramine (Tyr).

B: Effect of 5×10^{-5}M cocaine on the response to 3.65×10^{-4}M tyramine.

C: Effect of 5×10^{-6}M cocaine on the response to 3.65×10^{-4}M tyramine.

TABLE XII

Effect of Cocaine on Tyramine
ED_{50} (3.65×10^{-4}M)

Cocaine g/ml	Cocaine M	Mean Effect %↑ or %↓ ±S.E.	No. Expts.	p
5×10^{-7}	1.7×10^{-6}	+71 ± 20.6	8	<0.05
5×10^{-6}	1.7×10^{-5}	+86 ± 25.4	14	<0.05
5×10^{-5}	1.7×10^{-4}	+14.3 ± 27.4	10	N.S.
5×10^{-4}	1.7×10^{-3}	−36 ± 18.7	13	<0.05

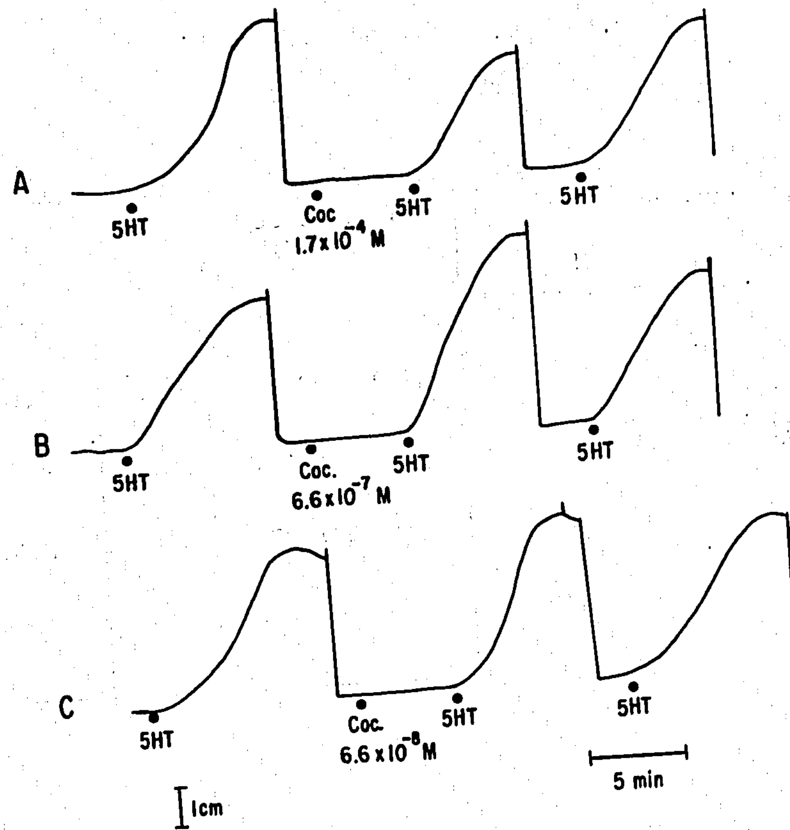


Figure 12. Effect of cocaine on the response to 5-hydroxytryptamine in the isolated umbilical artery.

A: Effect of 1.7×10^{-4}M cocaine (Coc) on the response to 1.26×10^{-7}M 5-hydroxytyryptamine (5HT).

B: Effect of 6.6×10^{-7}M cocaine on the response to 1.26×10^{-7}M 5-hydroxytryptamine.

C: Effect of 6.6×10^{-8}M cocaine on the response to 1.26×10^{-7}M 5-hydroxytryptamine.

TABLE XIII

Effect of Cocaine on 5-hydroxytryptamine
ED_{50} (1.26×10^{-7}M)

Cocaine g/ml	Cocaine M	Mean Effect %↑ or %↓ ± S.E.	No. Expts.	p
2×10^{-10}	6.6×10^{-10}	+44	2	---
2×10^{-9}	6.6×10^{-9}	+27 ± 15.3	3	N.S.
2×10^{-8}	6.6×10^{-8}	+32 ± 20.0	9	N.S.
2×10^{-8}	1.7×10^{-7}	+45	2	---
2×10^{-7}	6.6×10^{-7}	+29 ± 5.2	4	<0.05
5×10^{-7}	1.7×10^{-6}	+56 ± 12.3	4	<0.05
2×10^{-6}	6.6×10^{-5}	+18 ± 4.5	5	<0.05
5×10^{-6}	1.7×10^{-5}	+14 ± 3.0	4	N.S.
2×10^{-5}	6.6×10^{-5}	-5 ± 19.7	5	N.S.
5×10^{-5}	1.7×10^{-4}	-34 ± 15.7	6	<0.05
2×10^{-4}	6.6×10^{-4}	-36	2	---
5×10^{-4}	1.7×10^{-5}	-33 ± 3.4	4	<0.05

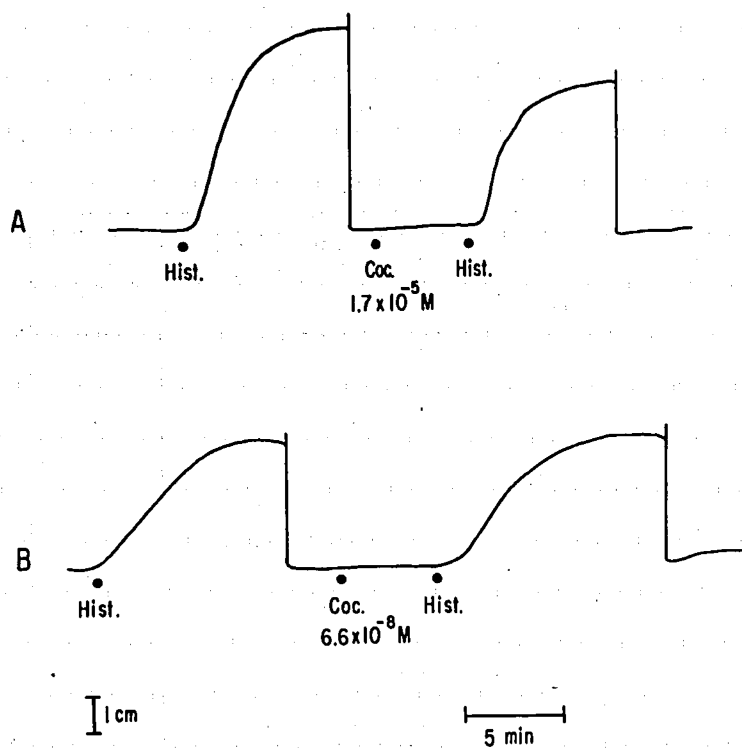


Figure 13. Effect of cocaine on the responses to histamine in the isolated umbilical artery.

A: Effect of 1.7 x 10^{-5}M cocaine (Coc) on the response to 1.8 x 10^{-6}M histamine (Hist).

B: Effect of 6.6 x 10^{-8}M cocaine (Coc) on the response to 1.8 x 10^{-6}M histamine.

TABLE XIV

Effect of Cocaine on Histamine
ED_{50} (1.8×10^{-6}M)

Cocaine g/ml	Cocaine M	Mean Effect %↑ or %↓ ±S.E.	Expts.	p
2×10^{-9}	6.6×10^{-9}	-15 ± 10.4	4	N.S.
2×10^{-8}	6.6×10^{-8}	-1 ± 4.1	4	N.S.
2×10^{-7}	6.6×10^{-7}	-9 ± 4.5	5	N.S.
2×10^{-6}	6.6×10^{-6}	-4	2	---
5×10^{-6}	1.7×10^{-5}	-8 ± 2.9	6	<0.05
2×10^{-5}	6.6×10^{-5}	-30	2	---
5×10^{-5}	1.7×10^{-5}	-13 ± 4.8	6	<0.05
2×10^{-4}	6.6×10^{-4}	-5	2	---

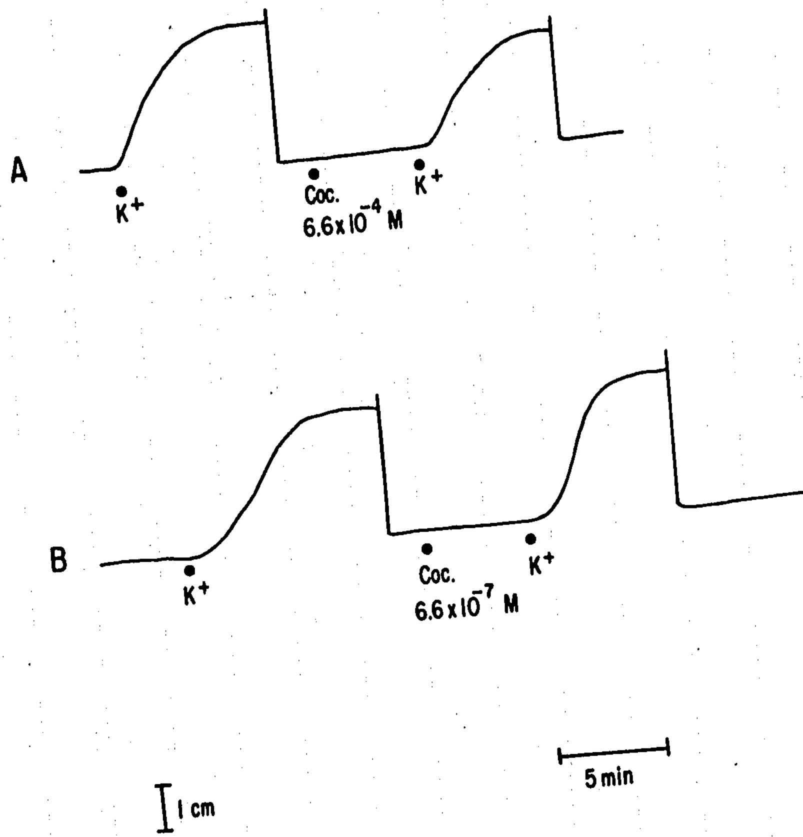


Figure 14. Effect of cocaine on the response to KCl in the isolated umbilical artery.

A: Effect of 6.6×10^{-4}M cocaine (Coc) on the response to 40mM KCl (K^+).

B: Effect of 6.6×10^{-7}M cocaine on the response to 40mM KCl.

TABLE XV

Effect of Cocaine on KCl

ED_{50} (40mM)

Cocaine g/ml	Cocaine M	Mean Effect %↑ or %↓ ±S.E.	No. Expts.	p
2×10^{-9}	6.6×10^{-9}	-7.5 ±	2	---
2×10^{-8}	6.6×10^{-8}	-4 ± 8.5	4	N.S.
5×10^{-8}	1.7×10^{-7}	+13 ±15.4	3	N.S.
2×10^{-7}	6.6×10^{-7}	-3.0 ± 1.5	4	N.S.
5×10^{-7}	1.7×10^{-6}	-4.0 ±13.8	8	N.S.
2×10^{-6}	6.6×10^{-6}	-3 ± 4.6	3	N.S.
5×10^{-6}	1.7×10^{-5}	+3 ± 3.8	7	N.S.
2×10^{-5}	6.6×10^{-5}	+1 ± 3.8	4	N.S.
5×10^{-5}	1.7×10^{-4}	-9 ± 4.6	5	N.S.
2×10^{-4}	6.6×10^{-4}	-15 ± 5.4	5	<0.05

was also significantly increased by cocaine, but the range of effective cocaine concentrations was more limited, 6.6×10^{-7} to 6.6×10^{-6}M (Table XIII).

At an equivalent concentration of cocaine (1.7×10^{-6}M), which causes maximal potentiation of the 5-hydroxytryptamine and $\frac{2}{3}$ maximal potentiation of the response to noradrenaline, it is seen that the degree of potentiation is significantly ($p<0.05$) greater for the response to noradrenaline.

Neither the histamine nor the potassium chloride induced contractures were increased by cocaine (Tables XIV and XV).

3. Effect of Phentolamine on Responses to Agonists

Table XVI and Figure 15 illustrate the effects of phentolamine on the ED_{50} responses to noradrenaline, tyramine and 5-hydroxytryptamine. Standard responses to the agonists were obtained, then the tissue was pretreated with various concentrations of phentolamine for 5 minutes and the agonist retested with the phentolamine present in the bath. There is a significant decrease in responses to agonists when the phentolamine concentration is 1.8×10^{-5}M or greater. Responses to noradrenaline and tyramine are significantly more sensitive to the antagonist action of phentolamine than are the responses to 5-hydroxytryptamine ($p<0.05$).

4. Effect of Propranolol on Responses to Agonists

Table XVII and Figures 16 and 17 illustrate the effects of propranolol on responses to various agonists. The experiments were

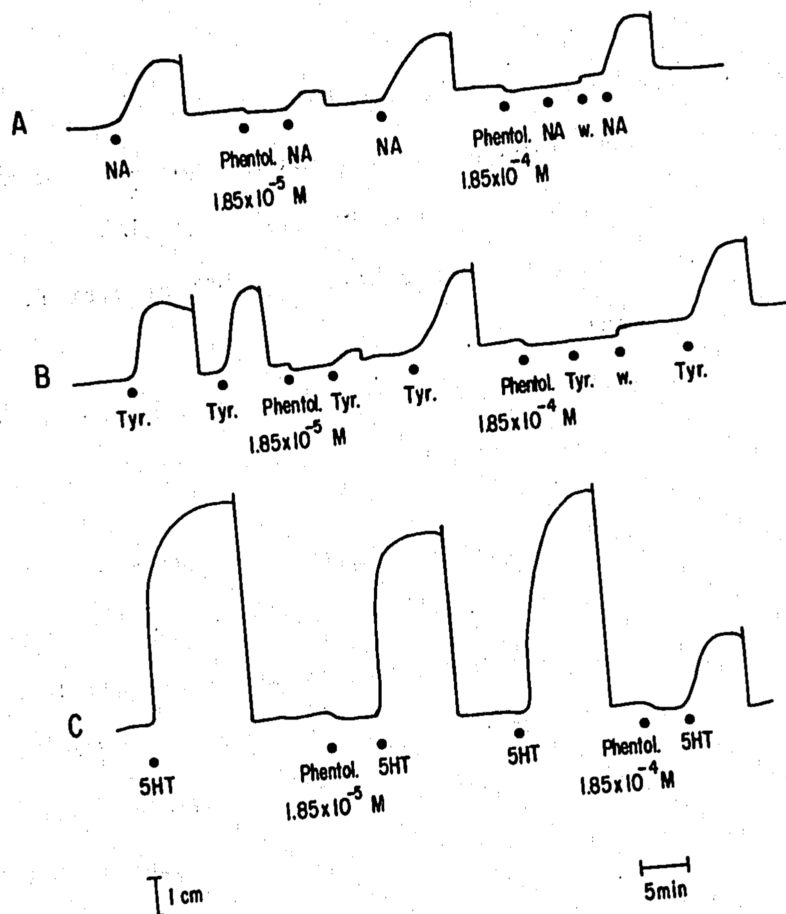


Figure 15. Effect of phentolamine on responses to noradrenaline, tyramine and 5-hydroxytryptamine.

A: Effect of 1.85 x 10^{-5}M and 1.85 x 10^{-4}M phentolamine (Phentol) on the responses to 3 x 10^{-5}M noradrenaline (NA).

B: Effect of 1.85 x 10^{-5}M and 1.85 x 10^{-4}M phentolamine on the responses to 3.65 x 10^{-4}M tyramine (Tyr).

C: Effect of 1.85 x 10^{-5}M and 1.85 x 10^{-4}M phentolamine on the responses to 1.26 x 10^{-7}M 5-hydroxytryptamine (5HT).

TABLE XVI

Effect of Phentolamine on Responses to Noradrenaline,

Tyramine and 5-hydroxytryptamine

Drug	Phentolamine g/ml	Phentolamine M	Mean Effect %↑ or %↓ S.E.	No. Expts.	p
Noradrenaline (5×10^{-6} g/ml or 3×10^{-5} M)	5×10^{-7}	1.8×10^{-6}	-3 ± 2.0	5	N.S.
	5×10^{-6}	1.8×10^{-5}	-63 ± 6.4	7	<0.05
	5×10^{-5}	1.8×10^{-4}	-100 ± 0	8	<0.05
	5×10^{-4}	1.8×10^{-3}	-100 ± 0	4	<0.05
Tyramine (5×10^{-5} g/ml or 3.65×10^{-4} M)	5×10^{-7}	1.8×10^{-6}	+7 ±26.8	3	N.S.
	5×10^{-6}	1.8×10^{-5}	-75 ±10.7	11	<0.05
	5×10^{-5}	1.8×10^{-4}	-100 ± 0	7	<0.05
	5×10^{-4}	1.8×10^{-3}	-100 ± 0	5	<0.05
5-HT (2×10^{-8} g/ml or 1.26×10^{-7} M)	5×10^{-7}	1.8×10^{-6}	-0.7 ± 5.2	3	N.S.
	5×10^{-6}	1.8×10^{-5}	-33 ± 9.7	8	<0.05
	5×10^{-5}	1.8×10^{-4}	-81 ± 6.2	5	<0.05
	5×10^{-4}	1.8×10^{-3}	-100 ± 0	4	<0.05

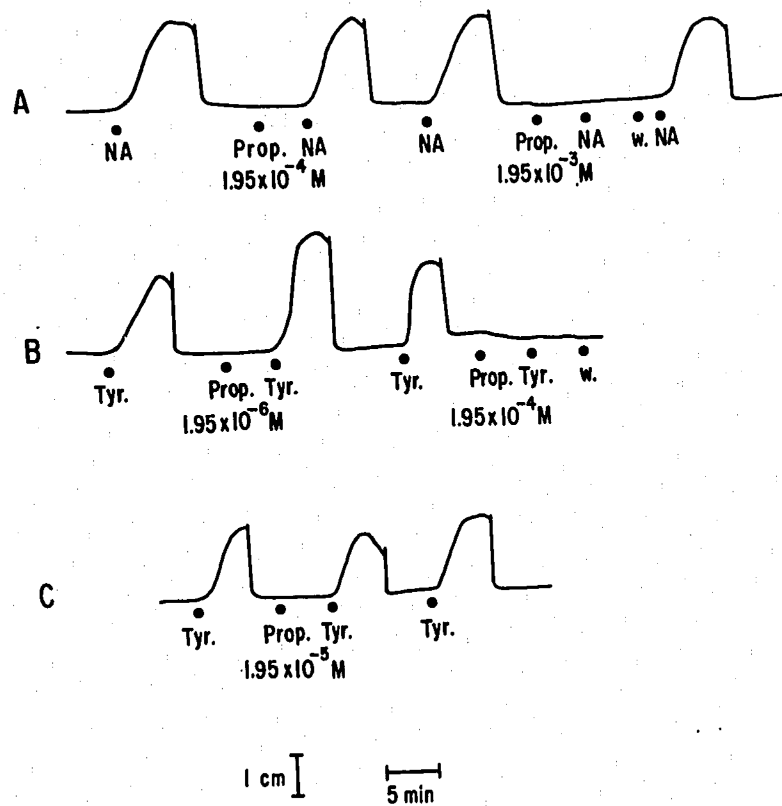


Figure 16. Effect of propranolol on the responses to noradrenaline and tyramine in the isolated umbilical artery.

A: Effect of 1.95×10^{-4}M and 1.95×10^{-3}M propranolol (Prop) on the responses to 3×10^{-5}M noradrenaline (NA).

B: Effect of 1.95×10^{-6}M and 1.95×10^{-4}M propranolol on the responses to 3.65×10^{-4}M tyramine (Tyr).

C: Effect of 1.95×10^{-5}M propranolol on the response to 3.65×10^{-4}M tyramine.

w. implies wash-out of the drug from organ bath

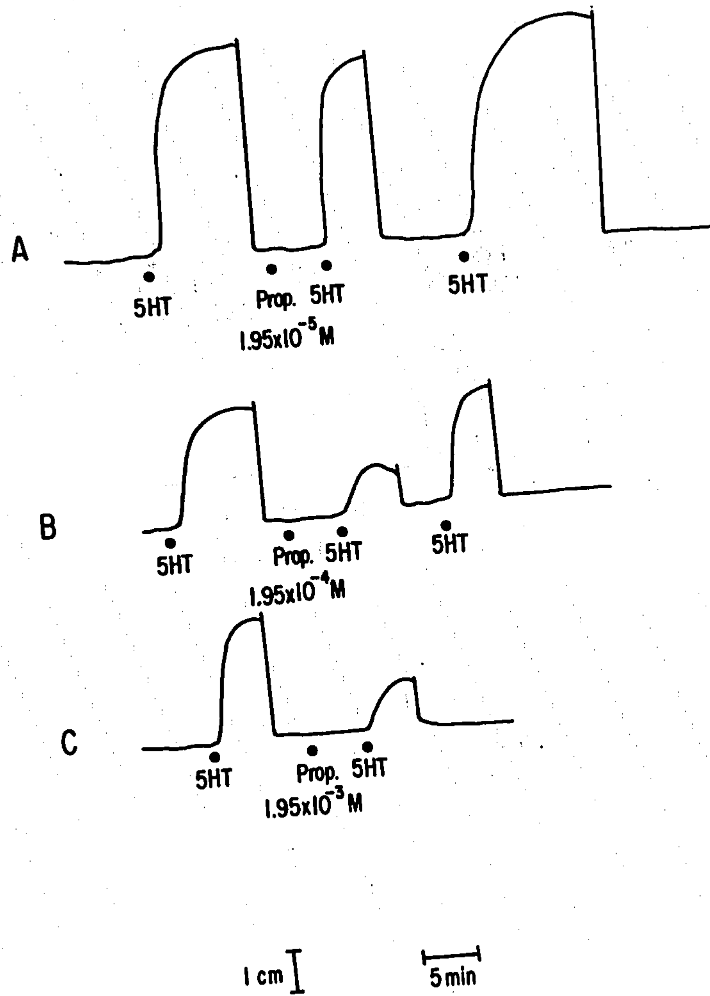


Figure 17. Effect of propranolol on responses to 5-hydroxytryptamine in the isolated umbilical artery.

A: Effect of 1.95×10^{-5}M propranolol (Prop) on the response to 1.26×10^{-7}M 5-hydroxytryptamine (5HT).

B: Effect of 1.95×10^{-4}M propranolol on the response to 1.26×10^{-7}M 5-hydroxytryptamine.

C: Effect of 1.95×10^{-3}M propranolol on the response to 1.26×10^{-7}M 5-hydroxytryptamine.

TABLE XVII

Effect of Propranolol on Responses to Noradrenaline, Tyramine and 5-hydroxytryptamine

Drug	Propranolol g/ml	Propranolol M	Mean Effect %↑ or %↓ S.E.	No. Expts.	P
Noradrenaline (5×10^{-6}g/ml or 3×10^{-5}M)	5×10^{-7}	1.95×10^{-6}	-7.5 ±	2	---
	5×10^{-6}	1.95×10^{-5}	+6 ± 4.8	4	N.S.
	5×10^{-5}	1.95×10^{-4}	+6 ± 6.9	4	N.S.
	5×10^{-4}	1.95×10^{-3}	-100 ± 0	3	<0.05
Tyramine (5×10^{-5}g/ml or 3.65×10^{-4}M)	5×10^{-6}	1.95×10^{-5}	+33.5±16.2	6	<0.05
	5×10^{-5}	1.95×10^{-4}	+33 ±90	4	N.S.
	5×10^{-4}	1.95×10^{-3}	-100 ± 0	3	<0.05
5-HT (2×10^{-8}g/ml or 1.26×10^{-7}M)	5×10^{-6}	1.95×10^{-5}	-9 ± 2.2	6	<0.05
	5×10^{-5}	1.95×10^{-4}	-63 ± 5.2	15	<0.05
	5×10^{-4}	1.95×10^{-3}	-68 ± 6.4	3	<0.05

designed in the same manner as those using phentolamine. It is noted that high concentrations of propranolol (1.95×10^{-3}M) cause a complete inhibition of responses to noradrenaline and tyramine, a lower propranolol concentration (1.95×10^{-5}M) significantly increased the response to tyramine but had no effect on the noradrenaline contracture, responses to 5-hydroxytryptamine are subject to a progressive decline in the presence of propranolol with a maximal reduction apparent at 1.95×10^{-4}M.

5. Effect of Noradrenaline on Induced Tyramine Responses

Table XVIII and Figure 18A illustrate the influence of noradrenaline on induced tyramine responses in the umbilical artery. Standard induced tyramine responses were obtained by the method described earlier, and then both noradrenaline and tyramine were administered to the tissue. It was found that threshold and subthreshold concentrations of noradrenaline, 3×10^{-7} to 3×10^{-6}M, significantly increased the induced-tyramine response. The effect of noradrenaline alone was determined following exposure to both noradrenaline and tyramine. It is noted that when the noradrenaline concentration is raised above threshold (*i.e.* $>10^{-5}$M) there is no significant ($p>0.05$) increase of the induced-tyramine response.

6. Effect of DPTC [2'(3 dimethylaminopropylthio)cinnamonilide hydrochloride] on Responses to 5-hydroxytryptamine, Noradrenaline and Tyramine

DPTC has been described as a fairly selective 5-hydroxytryptamine receptor antagonist (Paton, 1968) and has thus been employed in this

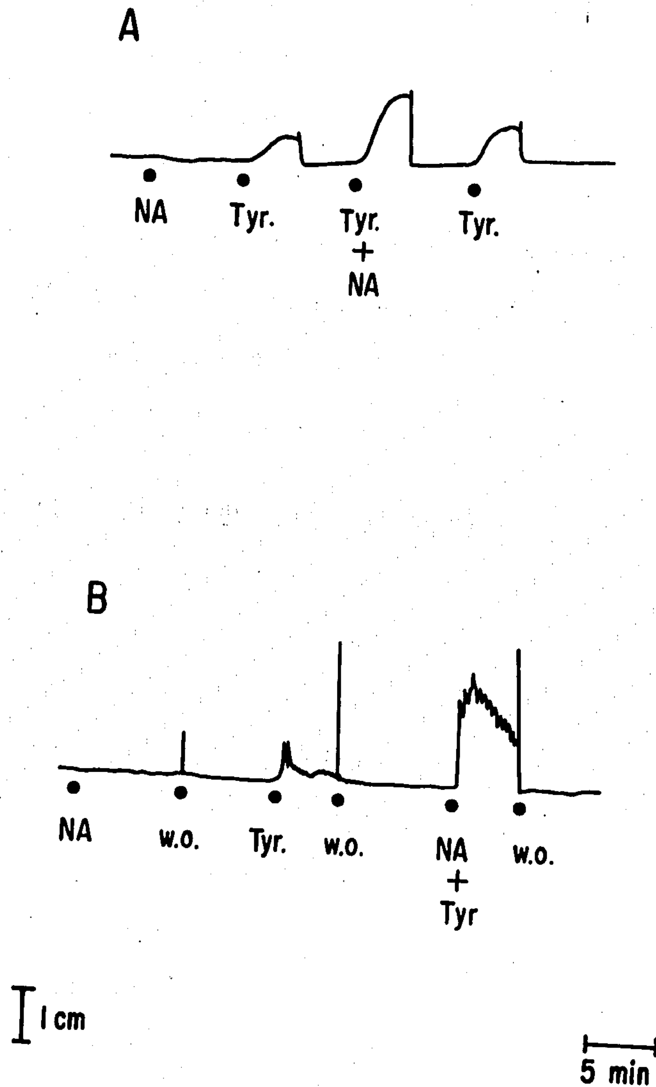


Figure 18. Effect of noradrenaline on the tyramine response in the isolated umbilical artery and the isolated guinea-pig vas deferens.

A: Effect of 3×10^{-7}M noradrenaline (NA) on the response to 3.65×10^{-4}M tyramine (Tyr) in the umbilical artery.

B: Effect of 3×10^{-11}M noradrenaline on the response to 3.65×10^{-5}M tyramine in the guinea-pig vas deferens.

w.o. implies the wash-out of the drug from the organ bath.

TABLE XVIII

Effect of Noradrenaline on Induced-Tyramine Response

Noradrenaline g/ml	Noradrenaline M	Mean Effect %↑ or %↓ ± S.E.	No. Expts.	p
5×10^{-8}	3×10^{-7}	+250 ± 93.1	6	<0.05
5×10^{-7}	3×10^{-6}	+121 ± 28.8	5	<0.05
5×10^{-6}	3×10^{-5}	+310 ±163	3	N.S.
5×10^{-5}	3×10^{-4}	+57 ± 28.5	5	N.S.

investigation to try and differentiate the effects of 5-hydroxytryptamine from those of noradrenaline and tyramine. Standard responses to the agonists were obtained and then the tissues were pre-treated with DPTC for 5 minutes and the agonist retested. It is shown in Table XIX and Figure 19 that responses to 5-hydroxytryptamine are significantly ($p<0.05$) more sensitive to DPTC than are the responses to noradrenaline and tyramine responses.

7. Cocaine Protection against Receptor Blockade by Phentolamine or Phenoxybenzamine

Table XX and Figure 20 illustrate the effect of cocaine against the blockade of α-adrenergic or 5-hydroxytryptamine receptors with phentolamine or phenoxybenzamine. Standard responses to noradrenaline and 5-hydroxytryptamine were obtained, the tissues were exposed to cocaine for 5 minutes, and then phentolamine or phenoxybenzamine for a further five minutes, and finally the agonist, either noradrenaline or 5-hydroxytryptamine, was added. It was noted that the concentrations of cocaine used, 1.7×10^{-4}M for noradrenaline and 6.0×10^{-5}M for 5-hydroxytryptamine, were those which had earlier been found to have no significant effect on responses to noradrenaline or 5-hydroxytryptamine (Tables XI and XIII). The concentration of phentolamine or phenoxybenzamine used were those which had been found to produce an approximate 50% reduction in the responses to the two agonists.

The results illustrated in Table XX suggest that cocaine cannot protect either noradrenaline or 5-hydroxytryptamine responses against phenoxybenzamine or phentolamine blockade.

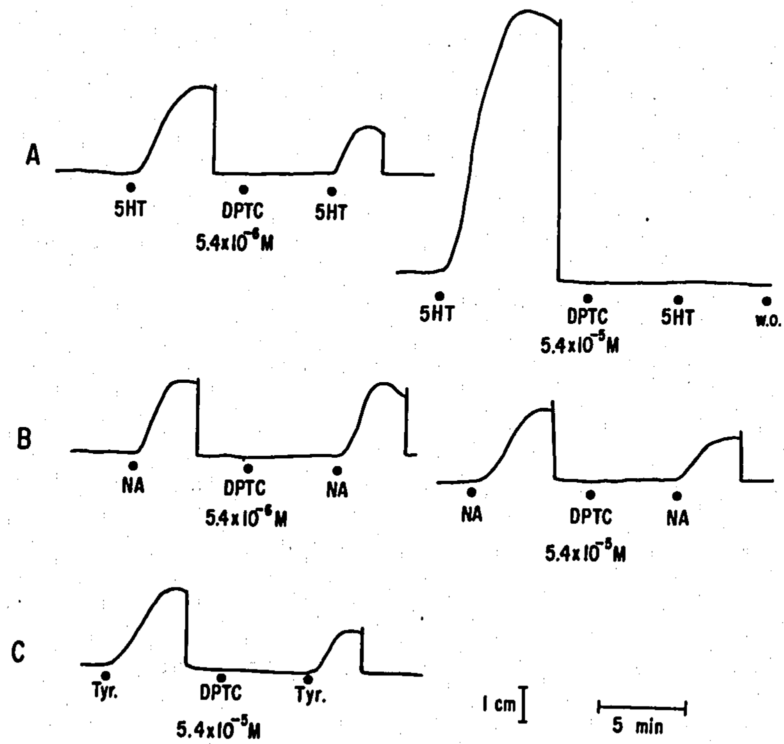


Figure 19. Effect of DPTC on the responses to 5-hydroxytryptamine, noradrenaline and tyramine in the isolated umbilical artery.

A: Effect of 5.4×10^{-6}M and 5.4×10^{-5}M DPTC on the responses to 1.26×10^{-7}M 5-hydroxytryptamine (5HT).

B: Effect of 5.4×10^{-6}M and 5.4×10^{-5}M DPTC on the responses to 3×10^{-5}M noradrenaline (NA).

C: Effect of 5.4×10^{-5}M DPTC on the response to 3.65×10^{-4}M tyramine (Tyr)

TABLE XIX

Effect of DPTC on Responses to 5-hydroxytryptamine, Noradrenaline and Tyramine

Drug	DPTC g/ml	DPTC M	Mean Effect %↑ or %↓ ± S.E.	No. Expts.	p
5-HT (2×10^{-8} g/ml or 1.26×10^{-7}M)	2×10^{-7}	5.4×10^{-7}	-2 ± 2.6	5	N.S.
	2×10^{-6}	5.4×10^{-6}	-42 ± 5.8	5	<0.05
	2×10^{-5}	5.4×10^{-5}	-100 ± 0	3	<0.05
Noradrenaline (5×10^{-6} g/ml or 3.0×10^{-5}M)	2×10^{-7}	5.4×10^{-7}	+4 ± 3.7	5	N.S.
	2×10^{-6}	5.4×10^{-6}	-4 ± 4.2	7	N.S.
	2×10^{-5}	5.4×10^{-5}	-35 ± 7.5	4	<0.05
Tyramine (5×10^{-5} g/ml or 3.65×10^{-4}M)	2×10^{-5}	5.4×10^{-5}	-41 ± 8.1	5	<0.05

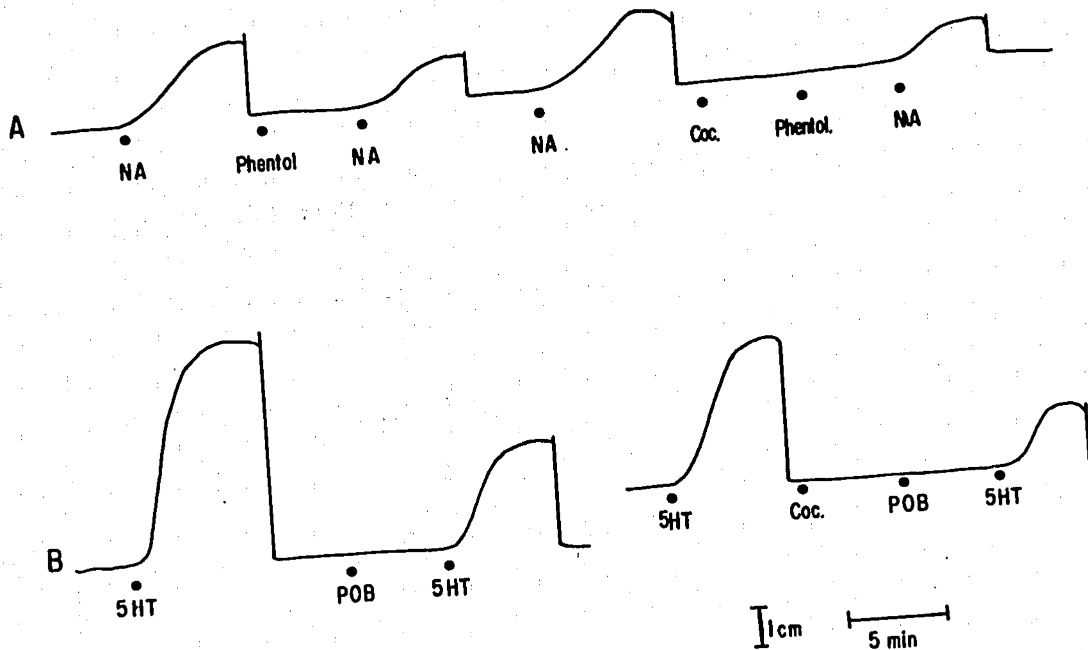


Figure 20: Effect of cocaine on receptor blockade produced by phentolamine and phenoxybenzamine in the isolated umbilical artery.

A: Effect of 1.7×10^{-4}M cocaine (Coc) on the phentolamine (Phentol), 7.2×10^{-6}M, induced α-receptor blockade of the response to 3×10^{-5}M noradrenaline (NA).

B: Effect of 6.6×10^{-4}M cocaine on the phenoxybenzamine (POB), 7.4×10^{-6}M, induced tyrptaminergic-receptor blockade of the response to 1.26×10^{-7}M 5-hydroxytryptamine (5HT).

TABLE XX

Cocaine Protection against Receptor Blockade by Phentolamine or Phenoxybenzamine

Blocking Agent g/ml	Blocking Agent M	Agonist	Mean Effect %↑ or %↓±S.E. plus cocaine	Mean Effect %↑ or %↓±S.E. minus cocaine	No. Expts.	P
Phentolamine						
2×10^{-6}	7.2×10^{-6}	Noradrenaline (5×10^{-6} g/ml or 3×10^{-5}M)	-38 ± 10.2	-43 ± 5.2	4	N.S.
5×10^{-6}	1.8×10^{-5}	5HT (2×10^{-8} g/ml or 1.26×10^{-7}M)	-34 ± 4.4	-36 ± 9.4	4	N.S.
Phenoxybenzamine						
5×10^{-8}	1.85×10^{-7}	Noradrenaline (5×10^{-6} g/ml or 3×10^{-5}M)	-56 ± 9.7	-52 ± 6.3	5	N.S.
2×10^{-7}	7.4×10^{-6}	5HT (2×10^{-8} g/ml or 1.26×10^{-7}M)	-50.6 ± 13.3	-51 ± 10.6	5	N.S.

IV. DISCUSSION

IV. DISCUSSION

A. WHOLE ORGAN PERFUSION

In the experiments that have been presented here the effect of cocaine on responses to noradrenaline, although significant, was quite small. von Euler (1938) demonstrated in the isolated perfused human placenta, that 2 mg of cocaine, injected 2-5 minutes before adrenaline, doubled the response to this catecholamine. Eliasson and Åström (1955) found that 3 mg of cocaine, injected 3-5 minutes prior to noradrenaline, caused a three-fold increase in the response to noradrenaline.

The tachyphylactic nature of the responses to tyramine obtained in the whole organ preparations support the theory that tyramine acts by releasing noradrenaline (Fleckenstein and Burn, 1933 ; Burn and Rand, 1958) the supply of which, on repeated administration of tyramine, gradually diminishes. This, of course, presupposes that sufficient releasable noradrenaline exists in the placenta and the umbilical cord. Table IV shows that low levels of noradrenaline can be detected in the placenta and the cord but whether these are sufficient or necessary for the response to tyramine was not demonstrated; however, noradrenaline was shown to restore the response to tyramine (see Figure 6 and Table II). That tyramine may have a direct action in the placental preparation, via α-adrenergic receptors or otherwise, is well documented for non-innervated (Takenaka, 1963; Somlyo et al., 1965; Gulati and Kelkar, 1971) as well as other tissues

(Luduena, 1963; Furchgott _et al._, 1963; Hudgins and Fleming, 1966; Bevan and Verity, 1967; Arthur and Fleming, 1968). Tyramine, also, may merely require a small amount of noradrenaline, at a subthreshold level, in order to induce a response. Thus, Nedergaard and Westermann (1968) found that a brief exposure of the stripped guinea-pig vas deferens to a threshold concentration of noradrenaline resulted in a large increase in the response to tyramine. This phenomena was not seen in the rat vas deferens (Barrett _et al._, 1968; Laporte _et al._, 1970). Kutschinsky _et al._ (1960), Lindmar and Muscholl (1961) and Nasmyth (1962) have also emphasized that low noradrenaline concentrations are sufficient for the production of a response to tyramine. A role of noradrenaline as an adjuvant or catalyst in the biophase for a direct response to tyramine was suggested by Fawaz (1961) and Murnaghan (1965). This present study has also indicated that subthreshold concentrations of noradrenaline can potentiate the response to tyramine in the isolated guinea-pig vas deferens (Figure 18B). The present results, and those of Nedergaard and Westermann (1968), using the guinea-pig vas deferens, could conceivably be explained by the uptake of sufficient noradrenaline during the brief exposure time to result in a tyramine induced release of noradrenaline which is far above the threshold concentration necessary for contraction.

B. NORADRENALINE ANALYSIS OF PLACENTA AND UMBILICAL CORD

The presence of small amounts of noradrenaline in the human placenta (0.045 μg/g) and umbilical cord (0.075 μg/g) is in disagreement

with the results of Davignon and Shepherd (1964) who found less than 0.005 μg/g noradrenaline in the human umbilical cord. The presence of noradrenaline is also rather surprising since the fluorescence microscopy studies reported here failed to detect any adrenergic nerves. However, Lachenmayer (1971) has found low levels of noradrenaline (0.03-0.06 μg/g) in extracts from the whole umbilical cord of the guinea-pig and the rabbit. The presence of this low level of noradrenaline could be explained in two ways: Firstly, there may be a sparse adrenergic nerve network in the umbilical cord. For instance, the lung has a noradrenaline concentration of 0.1 μg/g indicative of the sparse adrenergic nerve supply to this organ, whilst the average noradrenaline content of bovine splenic arteries and veins is 0.36 μg/g (von Euler and Lishajko, 1958). Secondly, the noradrenaline may be extraneuronally bound and may have originated from either some distant adrenergic nerve activity or from the adrenal medulla. Since the fluorescence histochemistry failed to demonstrate any adrenergic nerve endings, the second alternative seems most likely. The fact that noradrenaline is found associated with both the umbilical arteries and the veins suggests that it may originate from both the foetus and the mother. It has been shown that noradrenaline may cross the placenta (Baker, 1960; Beard, 1962; Sandler _et al._, 1963; Jacobson and Adamson, 1966). There is a marked increase in urinary noradrenaline and adrenaline during human labor (Zuspan _et al._, 1967; Zuspan, 1970; Goodall and Diddle, 1972) and noradrenaline and adrenaline have been detected in the amniotic fluid of the human foetus (Zuspan, 1970). The noradrenaline may also have originated from the foetal

adrenal glands as they have been shown to be capable of releasing noradrenaline (Comline and Silver, 1961; Comline and Silver, 1963, 1965; Hervonen, 1971) which is the predominant catecholamine in these glands (Shepherd and West, 1951; Hökfelt, 1952; Greenberg and Lund, 1961).

The *in vitro* demonstration of the relative lack of sensitivity of umbilical arteries to noradrenaline, compared, for instance, to the sensitivity of these vessels to 5-hydroxytryptamine, does not exclude the possibility that noradrenaline may be involved in the closure of the human umbilical arteries. Extrapolation of the results obtained *in vitro* to the situation which would be found *in vivo* is questionable. The difference between *in vivo* and *in vitro* results is well illustrated by the observation that in foetal lambs, before or during birth, *in vivo*, hypoxaemia caused umbilical vasoconstriction (Dawes, 1968). *In vitro* experiments, using isolated sheep placenta, demonstrated that hypoxaemia caused umbilical vasodilation (Lewis, 1968). *In vitro* experiments, using isolated human placenta or isolated umbilical arteries, have also demonstrated that hypoxaemia caused umbilical vasodilation (Nyberg and Westin, 1957; Panigel, 1962, Lewis, 1968; Bör and Guntheroth, 1970). The situation *in vivo* for the human is unclear (Dawes, 1968) and it is obvious that it would be dangerous to extrapolate the *in vitro* results to the *in vivo* condition. Dawes (1968) considers it possible that the closure of the extra-abdominal portion of the umbilical cord, *in vivo*, may be due to substances reaching the vessels through the blood stream, and could be explained by the release of catecholamines. Rather than a single cause for the closure of the umbilical arteries,

a large number of factors may interact in a complex manner to cause these cord vessels to constrict at birth. For example, prostaglandins may be a factor in closure of umbilical arteries. Prostaglandins are active in very low concentrations (10^{-9} - 10^{-8}M) in the human umbilical cord (Karim, 1967; Hiller and Karim, 1968), and can potentiate and inhibit the actions of certain agonists in other smooth muscle systems (Pickles *et al*., 1965; Clegg *et al*., 1966; Davies and Withrington, 1968; Davies *et al*., 1968; Nedqvist, 1970; Kadowitz *et al*., 1971a, 1971b). Recently prostaglandin-antagonists have been described by Sanner (1969), Fried *et al*. (1969), Eakins and Karim (1970), Eakins *et al*. (1970), Eakins *et al*. (1971) and their physiological role could now be tested.

C. FLUORESCENCE MICROSCOPY OF UMBILICAL TISSUE

This study has examined both normal and noradrenaline-incubated tissues and demonstrated that the human umbilical cord is not innervated by adrenergic nerve fibres. This conclusion is supported by the recent work of Walker and McLean (1971), who, using α-methylnoradrenaline and the Falck-Hillarp Method, also failed to detect adrenergic nerve fibres. Walker and McClean (1971) used α-methylnoradrenaline instead of the natural transmitter noradrenaline because the α-methyl derivative is monoamine oxidase (MAO) resistant. Our study has employed noradrenaline because the MAO content of the human umbilical cord is reported to be very low (Gennser and Studwitz, 1969). The lack of appreciable amounts of MAO in this tissue is consistent with the finding that the use of the MAO inhibitor parnate failed to enhance

noradrenaline uptake in the isolated perfused umbilical artery; these results are discussed later. Tissues pre-incubated with parnate and noradrenaline demonstrated no adrenergic nerve endings by fluorescence microscopy. An earlier histochemical study of adrenergic nerves in 20-24 week old human foetuses also revealed no innervation of the extra-abdominal umbilical cord beyond 1 cm from the foetus (Ehinger *et al.*, 1968).

It is possible that the short delay in freezing and sectioning the umbilical cord may have affected adrenergic nerve-endings present in the sample, however, it was shown that innervated vessels could still accumulate noradrenaline into the nerve endings after partial depletion of neuronal noradrenaline. Also, the adrenergic nerve ending of innervated tissues which have been left exposed to laboratory conditions for one hour or more, are still detectable by fluorescence histochemistry. If nerve endings were present but contained too little noradrenaline to be detected, then, following incubation with noradrenaline, they would have been visible by fluorescence microscopy. This statement is supported by the fact that Read and Burnstock (1970), using an identical technique, have shown that adrenergic nerve endings in foetal tissue can be detected after incubation with α-methyl noradrenaline whereas before treatment they were not visible.

In most vascular smooth muscle systems of adult animals, the terminal sympathetic effector plexus is confined to the adventitio-medial junction (Lever and Esterhuizen, 1961; Falck, 1962; Norberg and Hamberger, 1964), although the sheep carotid artery is an exception (Keatinge, 1966). Fluorescent nerve endings were found by Shibata *et al.*

(1971) in the media of the aorta of young (<1.3 kg) rabbits, but not of older rabbits (<3.5 kg). It is possible that the absence of an adventitia in the human umbilical artery (Maximow and Bloom 1952; Bailey, 1964) implies a lack of a nerve plexus; however, the lack of corroborative evidence makes this speculation tenuous. The extreme sensitivity of the histochemical method suggests that even a sparse adrenergic innervation in the umbilical cord would be detected. The amount of catecholamine needed for histochemical visualization seems to be very low, provided that it is concentrated in a specific location (de Champlain _et al._, 1970). Norberg and Hamberger (1964) estimated that less than 1 picogram (10^{-12} g) evenly distributed in the entire perinuclear cytoplasm, of average volume 10,000 - 20,000 μ^3, could be readily detected. Intense fluorescence was found when the estimated amount of noradrenaline in the adrenergic nerve endings was 0.4 x 10^{-6} µg (Norberg and Hamberger, 1964; Dahlström _et al._, 1966; Dahlstrom and Haggendal, 1966). Further emphasis as to the sensitivity of this technique is provided by the finding of Fuxe and Sedvall (1964) and Andén _et al._ (1966) where it was found that fluorescence was still readily detectable when noradrenaline content was reduced by 95% to levels as low as 10 ng/g. The limiting factor to detection of specific catecholamine fluorescence is the background fluorescence or auto-fluorescence, caused by tissue proteins. Corrodi and Jonsson (1967) found that in a model protein layer 10µ thick, primary catecholamines and 5-hydroxytryptamines could be demonstrated in concentrations of 0.001 to 0.005% (w/v) or 1-8 µg/g protein. In these studies, of course, the monoamines were evenly distributed throughout the protein and not

localised, as would be the case in nerve endings. The level of detection suggested by Corrodi and Jonsson (1967) would explain why no specific areas of fluorescence were detected in our study while in normal umbilical cords low levels of "extraneuronal" noradrenaline were detected by chemical analysis. If such levels of noradrenaline were localised in nerve endings it would be expected that the varicosities would have been detected by the histochemical technique.

On the basis of the studies performed here, the extra-abdominal portion of the human umbilical cord can be considered as being free of adrenergic nerves. The results here thus confirmed those of Walker and McClean (1971). Ellison (1971) has detected large bundles of acetylcholinesterase-positive fibers in the human umbilical cord. Acetylcholinesterase is an enzyme which is selectively present in nervous structures (Eränkö, 1967), however, the thiocholine technique, which was used by Ellison (1971), cannot distinguish between acetylcholinesterases and the nonspecific cholinesterases which are not necessarily associated with nervous structures. A further investigation into the presence of cholinesterases in the human umbilical cord utilizing an inhibitor of non-specific cholinesterases (Koelle and Gromadzki, 1966) would be valuable.

D. EFFECT OF DRUGS ON NORADRENALINE UPTAKE

1. Comparison of the Extraneuronal Uptake Process in Non-innervated Tissues to that in Innervated Tissues

In the present study it was found that the pre-incubation of

umbilical cord segments with phenoxybenzamine, cocaine or normetanephrine has no visible effect on the noradrenaline fluorescence as viewed by the fluorescence microscope. Experiments were also performed to show that cocaine, phenoxybenzamine and normetanephrine, at the concentrations used in this study, produced no fluorescence which could interfere with the interpretation of the experiments. It has been shown that 3-0-methylated or deaminated catecholamines do not react, or to only a very small degree, with the formaldehyde (Jonsson, 1967a, 1967b; Van Orden *et al.*, 1966). In our study, biochemical analysis of similarly treated umbilical arteries revealed that a significant decrease in noradrenaline uptake could be detected, the maximal decrease being that produced by 3.7×10^{-5}M phenoxybenzamine which caused a 29% decrease in noradrenaline uptake. Biochemical analysis also demonstrated that cooling the perfusion solution from 37°C to 0°C or 20°C significantly decreased noradrenaline uptake. Fluorescence microscopy, however, failed to detect these changes. The tissue to medium ratios that have been obtained for the extraneuronal process in innervated vascular smooth muscle systems (*cf*. Gillespie, 1968), although not accurately measured, do not seem to exceed 3:1; the tissue to medium ratios that we have demonstrated by biochemical analysis in the non-innervated human umbilical artery approach one. Thus, the extraneuronal process is more efficient in innervated systems; Burnstock *et al*. (1971) have reached the same conclusion. The decreases in noradrenaline uptake caused by phenoxybenzamine and normetanephrine in the umbilical artery were found to be much smaller than those encountered in innervated tissues, where the extraneuronal uptake of

noradrenaline is almost completely inhibited by these agents (Gillespie, 1969; Clarke et al., 1969; Lightman and Iversen, 1969; Gillespie et al. 1971; Burnstock et al., 1971). These differences cannot be explained on the basis of differences in efficiency of the extraneuronal uptake process is innervated and non-innervated tissues. Thus, using a noradrenaline perfusion concentration of 40 μg/ml, Gillespie (1968) and Gillespie et al. (1970) estimated the extraneuronal uptake of noradrenaline in cat spleen to be, at maximum, 120 μg/g. Using the umbilical artery, an extraneuronal noradrenaline uptake of not more than 40 μg/g tissue has been found in our experiments. Phenoxybenzamine was found to produce 95% inhibition of the extraneuronal uptake of noradrenaline by arterial smooth muscle (Gillespie, 1968) suggesting that the tissue level of noradrenaline under these conditions would be less than 6 μg/g. In the umbilical artery we have found that the inhibition does not exceed 30% suggesting that the extraneuronal uptake is of the order of 28 μg/g. Clearly the extraneuronal process in the non-innervated human umbilical artery is less sensitive to inhibition by phenoxybenzamine and normetanephrine.

No differences have been detected in the extraneuronal uptake of noradrenaline with or without phenoxybenzamine, normetanephrine, cocaine, or cold treatment when using fluorescence microscopy, but significant differences were found when the tissues were biochemically analysed for noradrenaline content. The reason these two techniques yielded different results is that fluorescence histochemical technique, despite its sensitivity and specificity, cannot detect relatively small changes in overall tissue fluorescence when one compares

different tissue sections. Such quantitative changes in catecholamine fluorescence are not detected because they are probably masked by differences in intensity of autofluorescence from one section to the next. Autofluorescence also tends to change with time, and catecholamine fluorescence, with continued excitation, fades with time. Jacobowitz and Brus (1971), who used a similar experimental design as that employed here (see METHODS), have concluded that normetanephrine, metanephrine, phenoxybenzamine, phentolamine and certain other drugs could markedly decrease extraneuronal noradrenaline uptake in the guinea-pig heart. In this instance, however, these inhibitors caused an almost complete inhibition of catecholamine fluorescence and thus such a change was readily detectable by fluorescence histochemistry. Since Burnstock _et al_. (1971) have used the same techniques for both innervated and non-innervated tissues and found the extraneuronal uptake process in non-innervated tissues is much less sensitive to inhibition by drugs than the process which occurs in innervated tissues, it is clear that the findings cannot be due to differences in technique. Avakian and Gillespie (1968), Gillespie (1968) and Gillespie _et al_. (1970) have used microspectrophotometer techniques which can detect changes in fluorescence intensity and, by using these techniques, they were able to detect changes in extraneuronal uptake after various treatments. Studies of catecholamine fluorescence using scanning microspectrophotometric techniques has been described by several authors (Caspersson _et al_., 1966; Gillis _et al_., 1966; Ritzen, 1966; Van Orden _et al_., 1966a, 1967; Jonsson, 1969; Lichensteiger and Langemann, 1969; Jonsson and Malmfors, 1970; Van Orden, 1970). However, this technique

also suffers from similar problems to those encountered in comparison of the reciprocal of the exposure time (see METHODS) and thus the measurements are only semi-quantitative (Ritzen, 1966; Van Orden *et al.*, 1966; Ploem, 1969).

Jonsson (1969) has suggested that it is advisable to correlate noradrenaline histochemical studies with chemical assays of the amine content. Biochemical analysis carried out in the course of our studies revealed that metaraminol, a potent neuronal uptake inhibitor (Iversen, 1967), did not affect the extraneuronal uptake of noradrenaline. Similar findings have been reported for the effect of metaraminol on extraneuronal uptake in innervated tissues (*cf.* Gillespie, 1968). The findings that metaraminol did not influence the noradrenaline uptake is consistent with the earlier conclusion that the human umbilical cord lacks adrenergic nerves; if adrenergic nerves were present some of the noradrenaline uptake would occur intraneuronally and should be significantly reduced by metaraminol.

The biochemical assay results obtained from perfusion of isolated umbilical artery strips with various concentration of noradrenaline (6×10^{-7}M to 2.4×10^{-4}M), provide evidence that this tissue can accumulate noradrenaline even when the perfusion concentration is as low as 6×10^{-7}M. After blocking MAO with parnate no changes were detected in the uptake of noradrenaline. Burnstock *et al.* (1971) have obtained similar results, using the non-innervated chick amnion and human umbilical cord, employing nialamide as the MAO inhibitor. Either MAO is not present in the tissue or, if MAO is present, it is not available to extraneuronal noradrenaline. Gennser and

Studwitz (1969) detected only low levels of MAO in the extra-abdominal portion of the human umbilical artery. When the innervated rabbit ear artery was pretreated with an MAO or COMT inhibitor, there was a significant increase in the noradrenaline uptake and the threshold level, at which noradrenaline uptake could be demonstrated histochemically, was lowered from 6×10^{-5} to 6×10^{-7}M (Burnstock et al., 1971). After blocking both MAO and COMT, Lightman and Iversen (1969) could detect accumulation by "$uptake_2$" in the isolated rat heart, at noradrenaline perfusion concentrations of 3×10^{-6} to 6×10^{-6}M.

Our study and that of Burnstock et al. (1971) have also indicated a similar level of detection, 6×10^{-7}M, in the non-innervated human umbilical artery. Eisenfeld et al. (1967a, 1967b) detected extraneuronal uptake of noradrenaline, or its metabolites, in the rat heart at a 3H-noradrenaline concentration of 3×10^{-8}M; an extraneuronal uptake site was confirmed by use of high concentrations of cocaine or immunosympathectomy. A much higher threshold of 6×10^{-5}M for extraneuronal noradrenaline uptake into the vascular smooth muscle of cat spleen was demonstrated by Avakian and Gillespie (1968) and a similar threshold level was detected by Burnstock et al. (1971) in the rabbit ear artery.

A cocaine sensitive component of extraneuronal noradrenaline uptake was reported by Kopin (1964) and Hughes et al. (1969). Our studies have indicated that cocaine can produce only a small inhibition (15%) of extraneuronal noradrenaline uptake into the human umbilical artery when used at high concentrations (3.3×10^{-4}M). Gillespie (1968) has also stated that cocaine has little or no effect on uptake of

extraneuronal noradrenaline into vascular smooth muscle. Clearly the mechanism for extraneuronal uptake of noradrenaline is considerably less sensitive to cocaine than the neuronal uptake of noradrenaline into the rat heart which is inhibited 50% when cocaine, at a concentration of 3.7 x 10^{-7}M, is present in the perfusion medium (Iversen, 1967).

2. The Mechanism and Function of Extraneuronal Uptake

It is not yet clear whether extraneuronal uptake of noradrenaline by smooth muscle cells, and other structures, has any physiological significance. The nature of the extraneuronal uptake is not known.

There is insufficient evidence to say whether extraneuronal noradrenaline is transported across the cell membrane by an energy-dependent active transport mechanism. It is not known whether the noradrenaline crosses the membrane against a concentration gradient, but Gillespie (1968) states that in the absence of intracellular binding of noradrenaline, passive diffusion across the membrane could not account for the accumulation of noradrenaline within the smooth muscle. Avakian and Gillespie (1968) and Gillespie et al. (1970) suggested that the process may involve an energy-dependent specific carrier since the process is inhibited by normetanephrine, phenoxybenzamine and ouabain. The results reported here have shown that 10^{-4}M ouabain can produce a 32% inhibition of the extraneuronal uptake of noradrenaline in the human umbilical artery, and Gillespie et al. (1970) demonstrated that 10^{-3}M ouabain produced a 50% inhibition of uptake in the cat spleen. The effect of ouabain, however, may not result from inhibition

of transport since the concentrations were much larger than those reported to inhibit membrane ATPase (10^{-5}M for 30 minutes, *cf*. Paton, 1971).

Eisenfeld *et al*. (1967a, 1967b) have suggested that extraneuronal uptake in innervated tissue may occur by facilitated diffusion alone. The claim was supported by the observation that noradrenaline was unlikely to be transported against a concentration gradient, but the process was inhibited by agents like phenoxybenzamine, normetanephrine and yet not affected by the potent "$uptake_1$" (*cf*. Iversen, 1967) inhibitors, cocaine or metaraminol.

The evidence for and against an active transport process or a facilitated diffusion process is clearly incomplete and thus further investigations are required. The experimental techniques employed by such workers as Bogdanski and Brodie (1969), Giachetti and Shore (1966), Iversen and Kravitz (1966), Gillis and Paton (1967) and Paton (1968a, 1971), have demonstrated a possible involvement of Na^+ and K^+ activated adenosine triphosphatase (membrane ATPase) in the transport of noradrenaline into adrenergic nerve endings. These techniques may be applicable to a further investigation of the extraneuronal noradrenaline uptake mechanism.

Uptake into the arterial smooth muscle is very sensitive to temperature change (Gillespie and Hamilton, 1967; Avakian and Gillespie, 1968; Gillespie *et al*., 1970) which is suggestive of active transport but is also consistent simply with chemical combination with a carrier. There is no evidence that the noradrenaline within the cells is retained against an electrochemical gradient. Our study indicated only

a small decrease in noradrenaline uptake when the temperature was 0-4°C. The calculated Q_{10} was 1.2, and this is not consistent with an active process in the human umbilical artery. Burnstock et al. (1971) have found no significant effect of reduced temperature on the uptake of noradrenaline into the non-innervated chick amnion and human umbilical artery. The pKa of noradrenaline has been estimated as approximately 9.78 (Lewis, 1954; Pratesi, 1963) and it would be expected that intracellular accumulation of noradrenaline may occur by diffusion. The limit of such a diffusion, without intracellular binding, would give a tissue to medium ratio of approximately 2.5 if one assumes an intracellular pH of 7.0 and an extracellular pH of 7.4. It is of interest that the tissue to medium ratios determined in this study seldom exceeded one; other workers (cf. Gillespie, 1968) have reported a tissue to medium ratio of approximately three. Hughes and Gillis (1968) have also indicated that the perfused rat lung concentrated noradrenaline to a level several times that of the perfusion medium. Thus a pKa dependent diffusion process can account for the intracellular/extracellular distribution of noradrenaline by the extraneuronal uptake process in the human umbilical artery and the non-innervated smooth muscle uptake may be an unspecialized diffusion process. Since accurate measurements of the extraneuronal intracellular/extracellular noradrenaline distribution in innervated tissues are not available this conclusion cannot, as yet, be extended to innervated smooth muscle. An unspecialized diffusion process cannot explain the inhibition of extraneuronal noradrenaline uptake by normetanephrine, metanephrine, phenoxybenzamine which is observed

in innervated tissues.

Our results and also those of Burnstock _et al_. (1971), indicate that whereas the initial loss of noradrenaline after extraneuronal uptake from the human umbilical artery is rapid, noradrenaline can still be detected after prolonged washing in noradrenaline-free Krebs. This finding suggests that some degree of intracellular binding may occur. The nature of such binding is, however, unknown.

Extraneuronal uptake may play an important role in transmitter inactivation (Eisenfeld _et al_., 1967a, 1967b; Lightman and Iversen, 1969). Phenoxybenzamine blocks formation of normetanephrine in hearts perfused with noradrenaline (Eisenfeld _et al_., 1967a; Iversen and Langer, 1969; Lightman and Langer, 1969; Jarrott, 1970) and phentolamine also has been shown (Eisenfeld _et al_., 1967a) to block extraneuronal uptake and reduce the formation of normetanephrine. Eisenfeld _et al_. (1967a, 1967b) suggest that normetanephrine produced locally, might serve to reduce the entry of excessive amounts of noradrenaline to extraneuronal intracellular sites where adrenergic receptors may be located. Thus, normetanephrine may not only be a metabolite of noradrenaline but might also serve to modulate the entry of the neurotransmitter to effector cells. The extraneuronal transport mechanism may either regulate access to the receptor or may be part of the receptor mechanism itself. Alternatively, the transport mechanism may function to increase access of noradrenaline to the intracellular enzymes that metabolize the catecholamine.

Certain extraneuronal structures such as fibroblasts and smooth muscle cells may be sites for noradrenaline uptake and metabolism by COMT. Certain tissues have been shown not to accumulate extraneuronal

noradrenaline and thus species and tissue differences may exist (Gillespie and Muir, 1970). Although such tissue differences may be due to variations in the activity of degradative enzymes (Lightman and Iversen, 1969), Jarrott (1970) failed to show any correlation between the extraneuronal metabolism of noradrenaline by perfused hearts and the activity of degradative enzymes in heart homogenates of a number of vertebrate species. Burnstock *et al*. (1971) did find a decrease in the threshold for fluorescence detection of noradrenaline uptake in the rabbit ear artery from 6×10^{-5}M to 6×10^{-7}M following inhibition of COMT and MAO. It would seem likely that the availability of degradative enzymes may affect extraneuronal uptake, at least in certain tissues. This question may be more accurately answered when the exact nature of the mechanism concerned with extraneuronal noradrenaline uptake is known.

The fact that both this present report and that of Burnstock *et al*. (1971) demonstrated that extraneuronal uptake of noradrenaline can be detected when the perfusion concentration of noradrenaline is as low as 6×10^{-7}M supports the suggestion that this uptake process can occur under physiological conditions.

E. ISOLATED TISSUE PREPARATION

1. General Pharmacology of the Isolated Tissue Preparation

Comparison of the effects of various agonists has shown that the isolated human umbilical artery is most sensitive to 5-hydroxytryptamine and this finding is in agreement with others (Somlyo *et al*., 1965;

Takenaka, 1963) who have used the same preparation. Both 5-hydroxytryptamine and histamine were capable of inducing contractions in preparations which were not affected by noradrenaline; this demonstrates that 5-hydroxytryptamine and histamine are unlikely to be acting via the α-receptor. The preparations responded initially to low concentration of synthetic oxytocin, angiotensin and acetylcholine but tachyphylaxis rapidly developed and eventually the tissue failed to respond to the drug even when the concentration was increased by up to 1000-fold. Tachyphylaxis to synthetic oxytocin and angiotensin has also been reported in the human umbilical artery preparation, by Somlyo *et al*. (1965).

An isopropylnoradrenaline-induced relaxation, or contraction, in umbilical vessels was not detected either at rest or during increased tension from a 5-hydroxytryptamine- or noradrenaline-induced contraction. The possibility that the β-adrenergic relaxation effect was masked by an effect of isopropylnoradrenaline on the α-receptors was eliminated by testing isopropylnoradrenaline on preparations in which the α-receptors had been blocked by phentolamine. Somlyo *et al*. (1965) and Gokhale *et al*. (1966) also failed to demonstrated functional β-receptors in the human umbilical artery, but, Ciuchta and Gautieri (1963) demonstrated a vasodilator response to isopropylnoradrenaline in the perfused human placenta. The vasodilation observed by Ciuchta and Gautieri (1963) may be due to an effect of isopropylnoradrenaline via β-receptors at sites other than in the umbilical arteries. If β-receptors mediate relaxation of the umbilical artery it would be anticipated that when they had been blocked, an increased response to

to sympathomimetics acting on both α and β-receptors would be observed. It was found, however, that the response to noradrenaline was not affected except at high propranolol concentrations (1.95×10^{-3}M) when the noradrenaline response was completely inhibited. In tissues where the interaction of adrenaline and propranolol was studied, similar results were obtained. An α-adrenergic blocking action of propranolol has also been observed in the rabbit aorta by Kohli and Ling (1967), and may be due to the nonselective actions of this compound at high concentrations (Mazurkiewicz-Kwilecki, 1970). A decline of the response to 5-hydroxytryptamine in the umbilical artery was also observed when this agonist was tested in the presence of increasing concentrations of propranolol. The induced tyramine response was also inhibited by high concentration of propranolol (1.95×10^{-3}M) but in the presence of 1.95×10^{-3}M propranolol the response to tyramine was significantly increased. This unexpected potentiation of the induced-tyramine response by propranolol is difficult to explain, but Innes and Karr (1971) found that another β-adrenergic blocking agent, pronethalol, potentiated the actions of sympathomimetics in the cat spleen; no reason for this potentiation was suggested. It would appear unlikely that there is a functional population of β-adrenergic receptors in the human umbilical artery.

It has been demonstrated, in the present study, that the isolated umbilical artery preparation is able to respond to tyramine either by a tachyphylactic mechanism, or after continued tyramine administration for 2-2½ hours when the non-tachyphylactic induced-tyramine response was produced (Figure 8). The induced-tyramine response could be described as an

auto-potentiation process where the response to tyramine is influenced by a preceding administration of tyramine. A similar auto-potentiation phenomena exists for the angiotensin response in the longitudinal smooth muscle of the guinea-pig ileum (Godfraind, 1968) and in helical strips of guinea-pig aorta (Godfraind, 1970). The explanation for this phenomena is, however, not clear. Leach and Fozard (1969) observed an auto-potentiation phenomena for the 5-hydroxytryptamine vasomotor response in the pithed rat. They suggested that a gradual, time-dependent, saturation of the 5-hydroxytryptamine tissue depots could account for the auto-potentiation. Possibly a similar mechanism, whereby a saturation of tissue tyramine depots is required before tyramine can elicit a response, operates in the human umbilical artery. The finding that phentolamine, in the concentration range 1.8×10^{-6} to 1.8×10^{-4}M, has a very similar blocking action on both responses to tyramine and noradrenaline suggests that the induced-tyramine response which is observed in the human umbilical artery preparation is due to a direct action of tyramine on the α-receptor.

The mechanism by which the induced-tyramine response is produced, however, is not clear. The sensitization of the response to tyramine by noradrenaline which was observed in both the human umbilical artery preparation and also the guinea pig vas deferens, is similar to the marked augmentation of the noradrenaline response produced by sub-threshold concentrations of 5-hydroxytryptamine in the isolated perfused central artery of the rabbit ear (de la Lande _et al_., 1966, 1967). de la Lande _et al_. (1967) suggested that the serotonin action is due to a sensitization of the smooth muscle membrane to excitation by

vasoconstrictor substances.

Åström and Samelius (1957) demonstrated that the vasoconstrictor action of both adrenaline and 5-hydroxytryptamine in the perfused human placenta could be blocked by phentolamine. Mancini and Gautieri (1964) found that phentolamine can partially block responses to 5-hydroxytryptamine in the perfused human placenta and suggested that this might be explained by an action of 5-hydroxytryptamine on the α-adrenergic receptor.

Innes (1962) suggested that 5-hydroxytryptamine acts via α-receptors in the cat spleen preparation, and examples exist where certain sympathomimetics have been postulated to act via 5-hydroxytryptamine receptors (Vane, 1960; Innes, 1963; Innes and Kohli, 1969; Innes, 1970). Since several of these investigations involved the use of receptor protection techniques it is possible that these interactions of sympathomimetics on 5-hydroxytryptamine receptors, and vice versa, only occur at high doses of the drugs (Kohli, 1969). Protection by high doses of a drug may therefore indicate a degree of affinity for a specific type of receptor, but does not necessarily indicate that interaction with these receptors is responsible for the action of the drug.

In view of the above findings, it was decided to investigate a possible action of tyramine and noradrenaline via 5-hydroxytryptamine receptors or 5-hydroxytryptamine acting via α-receptors, in the human umbilical artery. The effects of these three agonists were therefore determined with and without the presence of the α-adrenergic antagonist phentolamine, or the 5-hydroxytryptamine antagonist DPTC. The results

illustrate that increasing the phentolamine concentration from 1.8×10^{-6}M to 1.8×10^{-4}M causes, within the limits of S.E., the same degree of inhibition of responses to tyramine and noradrenaline. However, higher doses of phentolamine, were required to antagonize responses to 5-hydroxytryptamine than to antagonize the responses to noradrenaline. DPTC, in the concentration range 5.4×10^{-7} to 5.4×10^{-5}M, progressively decreased the tissue response to 5-hydroxytryptamine, but had much less effect on responses to noradrenaline and tyramine. These results further substantiate the view that noradrenaline and tyramine act via a receptor which is distinct from the one by which 5-hydroxytryptamine produces a response.

2. Effect of Cocaine on Responses to Agonists

The effect of cocaine on the uptake of noradrenaline into this preparations has been discussed earlier in this report. Cocaine, within the concentration range used in the isolated tissue experiments, can potentiate the effects of noradrenaline without having a significant effect on the uptake of noradrenaline. Potentiation of the response to noradrenaline by cocaine, in the perfused human umbilical artery, has been reported by Gulati and Kelkar (1971). Potentiation of responses to adrenaline, noradrenaline and 5-hydroxytryptamine by cocaine in the perfused human placenta was reported by von Euler in 1938, and Eliasson and Åström (1955) respectively.

The effect of cocaine on tyramine and 5-hydroxytryptamine uptake in the human umbilical cord is not known, but Dyer (1970b) has shown that 3.3×10^{-5}M cocaine inhibits, by 30%, the uptake of 3H-5-hydroxy-

tryptamine into the sheep umbilical artery. The same concentration of cocaine also increased by 1080% the response to 5-hydroxytryptamine in this tissue. Mast cells are present in the human umbilical cord (Sundberg *et al*., 1954) and they may take up 5-hydroxytryptamine. Day and Stockbridge have reported that cocaine, 3.3×10^{-8}M, inhibited 5-hydroxytryptamine uptake into mast cells by 90-96%. It seems unlikely that a 30% reduction of 5-hydroxytryptamine uptake into mast cells can be the only factor contributing to the 1080% increase of the response to 5-hydroxytryptamine in the sheep umbilical artery and thus, an effector cell action of cocaine could also be dominant in this tissue. This problem may be answered when the site(s) for 5-hydroxytryptamine uptake in the sheep umbilical artery are known. Cocaine has been reported (Dyer, 1970a) to cause only slight potentiation of the response to noradrenaline in the sheep umbilical artery; the effect of cocaine on noradrenaline uptake in this tissue has not been investigated.

The present investigation has also shown that the responses to 5-hydroxytryptamine were potentiated by pretreatment of the human umbilical artery with cocaine. The degree of potentiation, however, was less than that observed with the response to noradrenaline. The range of cocaine concentration which potentiated the response to 5-hydroxytryptamine was smaller than that which affected the noradrenaline; thus at a cocaine concentration of 1.7×10^{-5}M there is no significant change in the response to 5-hydroxytryptamine whereas that to noradrenaline is greatly increased. The incomplete data suggests that the induced-tyramine response is potentiated by a similar range of cocaine concentrations; however, the degree of potentiation, in all cases, was less.

Potentiation of responses to tyramine by cocaine in the isolated perfused umbilical artery has been reported by Gulati and Kelkar (1971).

Our results have shown that the responses to histamine and potassium chloride were not significantly potentiated by any concentration of cocaine. As noted earlier, the responses to noradrenaline, tyramine, 5-hydroxytryptamine, histamine and potassium chloride could all be decreased by increasing the cocaine concentration. Responses to noradrenaline and tyramine were decreased significantly when the cocaine concentration was raised to 1.7×10^{-3}M, 5-hydroxytryptamine and potassium chloride contractures were significantly reduced when the cocaine concentration was raised to 1.7×10^{-4}M and 6.6×10^{-4}M respectively. The histamine contractures was significantly reduced at cocaine concentrations of 1.7×10^{-5}M or more. This inhibitory action of cocaine on smooth muscle contraction may be due to a stabilizing action on the muscle membrane similar to that reported for other local anaesthetics (cf. Feinstein and Paimre, 1969).

3. Protection Experiments

Vohra (1969) failed to detect protection by cocaine (6.6×10^{-4}M) against blockade with phenoxybenzamine (1.8×10^{-7}M) of either the α-adrenergic or cholinergic receptors in reserpinized rat vas deferens. Cocaine, however, was found to cause contraction of tissues which had been protected by noradrenaline (6×10^{-4}M) against phenoxybenzamine blockade.

Innes and Karr (1971) recently demonstrated in the cat spleen

that phentolamine could protect against cocaine potentiation of the response to noradrenaline. Cocaine was not able to potentiate the response to noradrenaline when phentolamine had been washed out of the tissue i.e. when the α-receptors were no longer blocked. Loss of the antagonist action of phentolamine was indicated by the reappearance of control responses to noradrenaline in phentolamine-treated spleen strips.

Receptor protection experiments, critically evaluated by Waud (1962, 1968) and Triggle (1971), have been employed to indicate a common interaction site for ligands (Furchgott, 1954, 1966); however, in such experiments it is common practice to use a high concentration of the protecting agent and, at this concentration, one cannot conclude that interaction is merely with the receptor. Phenoxybenzamine, a 2-halogenoethylamine, is often used as the antagonist in receptor protection experiments; however, this agent, and those related to it, are capable of affecting many other sites besides the α-adrenergic receptor (Moran et al., 1967; Yong and Marks, 1969). The reversible α-receptor antagonist phentolamine affords better and more consistent protection against inactivation by the irreversible antagonists (Lewis and Miller, 1966; Patil et al., 1968). It is not known, however, whether phentolamine acts at the same site as noradrenaline, the noradrenaline-recognition site, or whether it produced competitive-antagonism of the α-response by an action at another distinct site which is related to the α-receptor in some manner.

With the above reservations in mind, an attempt was made, in the umbilical artery strip, to prevent or decrease by cocaine pretreatment

the receptor blockade against noradrenaline or 5-hydroxytryptamine contractures produced by phentolamine or phenoxybenzamine. If some significant protection had been produced by pretreatment with cocaine then one could suggest that cocaine can interact at a site which is affected by phentolamine and/or phenoxybenzamine. Of course, this would not imply that cocaine actually acts at the α-adrenergic or the 5-hydroxytryptamine receptor. It should be noted that the cocaine concentration used for protection were those which had been shown to cause neither significant potentiation nor significant depression of the response to noradrenaline or 5-hydroxytryptamine and thus interpretation of the data is not complicated by these considerations. The concentrations of blocking agents used were those which caused approximately 50% blockade of noradrenaline or 5-hydroxytryptamine contractures. As noted there was no significant protection by cocaine against the blocking action of phentoalmine or phenoxybenzamine on the responses to noradrenaline or 5-hydroxytryptamine. It would thus appear that cocaine has little affinity for the sites occupied by phentolamine or phenoxybenzamine, and cannot prevent the α-adrenergic or tryptaminergic blocking action of these compounds.

F. EVALUATION OF THE "COCAINE PARADOX"

Burn and Tainter (1931) adopted the term "cocaine paradox" to describe the action of cocaine on adrenergic mechanisms in smooth muscle systems. Subsequent publications (Trendelenburg, 1959, 1963, 1966; Iversen, 1967) have suggested that at least part of the cocaine-induced supersensitivity was due to an inhibition of neuronal catecholamine

uptake. The degree to which this blockade of neuronal uptake can account for supersensitivity to noradrenaline has never been directly shown due to technical difficulties; however, a correlation between inhibition of noradrenaline uptake by cocaine and the development of supersensitivity has been suggested by several authors (Barnett, 1968; Foster, 1968). A similar correlation has also been suggested by Brimijoin et al. (1970) for the onset of denervation supersensitivity to noradrenaline in the cat spleen.

The present investigations have shown that the human umbilical artery is devoid of adrenergic nerves and "extraneuronal" uptake of noradrenaline is only affected by cocaine when cocaine is used at high concentrations. A concentration of cocaine having no effect on noradrenaline uptake can potentiate the response of the tissue to that same concentration of noradrenaline. It is clear that cocaine, in this tissue, is acting via a mechanism which is not related to blockade of noradrenaline uptake and which is probably directly on the effector cell. This direct action on the effector cell might be equivalent to the "postsynaptic" action which has been suggested to occur in innervated systems (Nakatsu, 1968; Nakatsu and Reiffenstein, 1968; Kalsner and Nickerson, 1969b; Kasuya and Goto, 1968; Varma and McCullough, 1969; Shibata, 1971).

It has been shown that responses to tyramine and 5-hydroxytryptamine are potentiated, and the increase of the response to 5-hydroxytryptamine demonstrates that the action of cocaine is not entirely specific for the α-adrenergic response; however, responses to histamine and KCl are not significantly altered by cocaine. Kalsner and Nickerson (1969b)

have demonstrated that cocaine potentiates responses to histamine without affecting those of 5-hydroxytryptamine in the rabbit aorta, and Shibata et al. (1971), using the same preparation, have shown that the respnses to KCl were potentiated by cocaine. Nonspecific cocaine potentiation of agents other than catecholamines has also been reported by Kasuya and Goto (1968) using the rat vas deferens, and by Dzoljić et al. (1970) using the terminal guinea-pig ileum. Greenberg and Long (1971), however, observed no potentiation of responses to KCl, $BaCl_2$ or acetylcholine by treatment with cocaine in the reserpinized rat vas deferens preparation. It is possible that this non-specific potentiation may be due to the potentiation of released endogenous noradrenaline (Douglas and Rubin, 1964; Kiran and Khairallah, 1969). The potentiation by cocaine of the responses to 5-hydroxytryptamine which were observed in the present study were not, however, due to the potentiation of released endogenous noradrenaline since several preparations responded to 5-hydroxytryptamine and not to noradrenaline. Nakatsu (1968) and Nakatsu and Reiffenstein (1968) found that the process by which cocaine potentiates smooth muscle contraction of the rat vas deferens is involved with excitation by noradrenaline and does not affect the response to methacholine.

Potentiation of noradrenaline responses in adrenergically innervated tissues, however, is not universal and thus Paton (1958b) reported that contractions to submaximal concentration of noradrenaline were not significantly altered by cocaine in the isolated oestrogenized rat uterus. It has also been found that although cocaine can potentiate

certain excitatory effects of catecholamines, the inhibitory effects of catecholamines, such as those on the uterus and intestine, seem to be unaffected (Burn and Tainter, 1931; Gruber, 1936; Roszkowskew and Koelle, 1960; Stafford, 1963; Andén *et al.*, 1964; Govier, 1969). The depressor response to adrenaline, following α-receptor blockade is also not potentiated by cocaine (Maxwell, 1959). The inhibitory effects of noradrenaline in the guinea-pig trachea, however, are potentiated by cocaine (Foster, 1967, 1968; Chahl and O'Donnell, 1967, 1971). The large size of the synaptic cleft distance in the guinea-pig small intestine (>1000Å, *cf*. Bennett and Rogers, 1967) has been suggested by Govier *et al*. (1969) to explain the lack of cocaine potentiation of catecholamine relaxation responses in this tissue. The synaptic cleft in the cat nictitating membrane, where cocaine can potentiate the responses to noradrenaline by a factor of from 23 to 50 (Fleming and Trendelenburg, 1961; Trendelenburg, 1965) is between 200 and 600Å (Esterhuizen *et al.*, 1968). Extraluminal noradrenaline in the isolated central artery of the rabbit ear is also potentiated to a greater degree than is intraluminal noradrenaline (de la Lande *et al.*, 1967). However, the work of Bevan and Verity (1967), using surgically denervated rabbit aorta, suggests that the synaptic cleft size may not be the prominant factor involved in cocaine-induced supersensitivity to noradrenaline in this tissue since cocaine potentiated noradrenaline in the surgically denervated rabbit aorta. The use of non-adrenergically innervated human umbilical artery in our study has also clearly shown that the size of the synaptic cleft is not the only factor which can affect the cocaine supersensitivity

phenomenon. Trendelenburg (1968) stated that there was no compelling reason for the view that cocaine must have only one site of action, and the results presented here support this claim. It would seem reasonable to suggest that two basic mechanisms can explain cocaine-induced supersensitivity:

Firstly, cocaine can block noradrenaline uptake across the membrane of the adrenergic nerve ending and thus increase the local concentration of noradrenaline; this can be regarded as the pre-synaptic action. The relation of this block of neuronal uptake to supersensitivity is probably dependent upon the density of innervation of the tissue, and tissues with an extensive and abundant adrenergic innervation, like the nictitating membrane, are most likely to be affected by this action of cocaine.

Secondly, cocaine has a postsynaptic action on the smooth muscle cell and this action is more obvious in sparsely innervated or non-innervated tissues. These postsynaptic changes possibly have at least two components: one change is selective towards the excitatory α-adrenergic receptor, whilst the second change is relatively non-selective and the degree to which individual agonists are changed is tissue and species dependent. Of related interest are the nonspecific smooth muscle supersensitivities to catecholamines, acetylcholine and K^+, which are produced by reserpine and decentralization (Trendelenburg and Weiner, 1962; Hudgins and Fleming, 1966; Westfall, 1970), and may be related to an increased tissue affinity for Ca^{++} (Green and Fleming, 1967; Garrett and Carrier, 1971).

Unfortunately, it is difficult to differentiate clearly between

a selective action of cocaine on the α-adrenergic receptor and a non-selective increase in the sensitivity of the smooth muscle to other agonists. The existence of two such actions, however, would suggest an explanation for the preferential effect of cocaine on responses to noradrenaline in tissues where the primary action of cocaine cannot be due to blockade of noradrenaline uptake.

It has been suggested (Reiffenstein, 1968; Innes and Karr, 1971) that cocaine may act near the receptor to alter noradrenaline receptor combination, and the hypothesis stated was that cocaine caused an allosteric alteration of the α-adrenergic receptor. Although there is no direct evidence for this hypothesis in cellular drug-receptor systems, it can be considered to represent a close analogy with the current concepts regarding allosteric interactions with enzyme systems (cf. Monod et al., 1963). Triggle (1971) has suggested that at least a part of the diverse pharmacological activity of many 2-halogeno-ethylamines can be best attributed to a relatively nonspecific binding at a variety of macromolecular sites producing antagonism by nonspecific allosteric interactions.

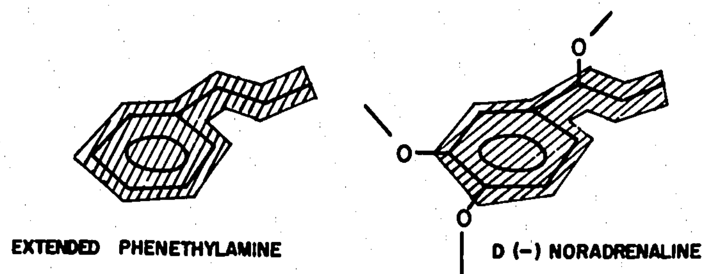
The postsynaptic action of cocaine can be explained by an allosteric alteration of receptors which results in an increased sensitivity to certain agonists. In most adrenergically innervated tissues containing α-excitatory receptors the allosteric modifying action may selectively alter the sensitivity of α-receptor to stimulants and also nonselectively alter the smooth muscle sensitivity to other agonists. Such a mechanism adequately explains the greater potentiation of ED_{50} responses to noradrenaline compared to agonists in many

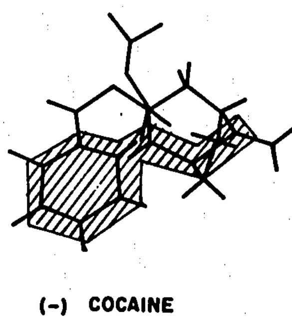
adrenergic systems including the human umbilical artery. Evidence to support this model is, unfortunately, indirect and thus this suggestion is, at present, pure speculation.

As described in the INTRODUCTION, Moran et al. (1970) have suggested that a common site of 2-halogenoethylamine action in the smooth muscle system may be that concerned with Ca^{++} mobilization to the excitation-contraction coupling process and may be similar, although not necessarily identical, for each group of activator ligands. Two sites were postulated to exist whereby 2-halogenoethylamines could antagonize noradrenaline. One site represents a Ca^{++} binding/mobilization site on the cell membrane essential to excitation-contraction coupling; the second site may represent the noradrenaline recognition site. Several authors have postulated, but not directly shown, a calcium-dependent component of cocaine supersensitivity. Carrier and Shibata (1967), Shibata (1969a) and Shibata et al. (1971) have suggested that an increased membrane permeability to calcium may be a common mechanism to explain supersensitivity phenomena. Greenberg and Innes (1968) have also speculated that, in the presence of cocaine, the cat spleen utilizes less extracellular Ca^{++}. Daniel and Wolowyk (1966) have suggested that cocaine induces labilization of Ca^{++} from the cell membrane of rat uterus. Nakatsu (1968) has also suggested that cocaine may prime a labile Ca^{++} pool which is utilized specifically by the α-receptor and from which Ca^{++} can be released by α-receptor stimulation. Possibly, cocaine can selectively facilitate the mobilization of Ca^{++} from a binding site which is linked to the noradrenaline site. This Ca^{++} binding site is linked to the noradrenaline-recognition site in

such a manner that Ca^{++} is not made available for contraction until the noradrenaline-site is activated. Such an action for cocaine would be, effectively, the opposite to that suggested for 2-halogenoethylamines by Moran et al. (1971). Cocaine may also influence the action of other receptors, possibly by a similar, but less selective, action, and species and tissue differences in the cocaine binding site(s) may explain the variability in results obtained for activator ligands in different preparations. Similar differences for antagonist binding sites have been suggested by Bevan and Osler (1965), Gurchgott (1970), and Janis and Triggle (1971). Clearly further investigations are required to elucidate the action of cocaine on smooth muscle.

The site of action of cocaine is not known, but Maxwell et al. (1968, 1969) have reported that certain tricyclic antidepressants such as desmethylimipramine (DMI) have conformational similarities to the trans-staggered conformation of β-phenethylamine. Maxwell et al. (1970) have also suggested that cocaine can also adopt a similar conformation to noradrenaline and this might explain its action on the "amine-pump receptor" (Figure 21). The absolute configuration of cocaine has been determined (Hardegger and Oht, 1955) but whether such a conformation confers upon cocaine an action at the α-adrenergic receptor is not known. Interpretation of structural-activity relationships are complicated by the unknown factor of drug availability in the biophase and the consequent influence of this factor upon events at the receptor. The protection experiments described in this present study and by Vohra (1969) suggest that cocaine has little affinity for the site occupied by phentolamine, phenoxybenzamine or noradrenaline,





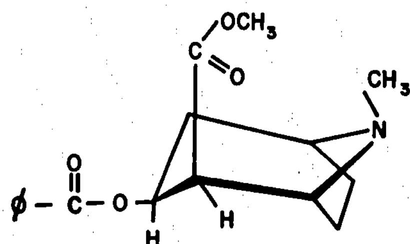


Figure 21: The configuration relationships between phenethylamine, D(–)noradrenaline, and (–) cocaine.

but this does not mean that cocaine cannot alter the noradrenaline recognition site or, for that matter, other receptor sites of the cell. An analogous situation is that which has been described by Koshland (1963):

> "The substrate is like a spider at the centre of the web. It can change the conformation near the centre and start a pertubation which will cause a change in a far distant corner of the web. It follows, of course, that a change in a far distant corner of the web may be felt by the spider at the centre."

Unfortunately, virtually nothing is known of the chemical constitution of receptors in excitable tissues. It is believed that these entities are composed of protein or lipoprotein components that are integrated into the membrane structure (Bloom, 1966; Cuthbert, 1967; De Robertis, 1967, 1971; DePlazas and De Robertis, 1972; Ochoa _et al_. 1972). Drug receptors are characterized not by their composition, shape, size or location, but by the molecules that bring about a physiological response which has been identified with the activity of the receptor. It is apparent that many of the neurotransmitters and their analogs are small, relatively flexible molecules, the conformation of which, as receptor-bound species, need bear no relationship to the preferred conformation in the aqueous or crystalline state (Ridley _et al_., 1969; Robinson _et al_., 1969). The drug receptor itself may also possess flexibility (Portoghese, 1970).

The problem of the "cocaine paradox" has been partially solved. In the human umbilical artery the action of cocaine must be "postsynaptic" since there are no adrenergic nerves present in this system. Our study shows that the effect of cocaine in this system is not mediated

by blockade of noradrenaline uptake into the effector cell. The exact role for the blockade of neuronal or effector cell, noradrenaline uptake by cocaine in other systems is not clear but it is of interest that Dyer (1970b) demonstrated an eleven-fold increase for the response to 5-hydroxytryptamine in the sheep umbilical artery preparation. If the sheep umbilical arteries are also devoid of adrenergic innervation, as has been shown in the present study for the human umbilical artery, then this implies that the postsynaptic action of cocaine may, in the sheep umbilical artery preparation, be very important to the supersensitivity phenomenon. It should, however, be restated that Dyer (1970b) demonstrated that a 30% reduction in 5-hydroxytryptamine uptake was associated with this increase in the response to 5-hydroxytryptamine. A knowledge of the location for 5-hydroxytryptamine uptake in the sheep umbilical artery would tell us whether this relatively small reduction in uptake is the cause of the large increase in the response to 5-hydroxytryptamine. In order to further explore the nature of the postsynaptic action of cocaine three basic questions are dominant:

1. Where on the postsynaptic cell does cocaine act?
2. What changes can cocaine induce in the smooth muscle cell?
3. How do these changes result in an increased reponse to certain smooth muscle agonists?

In order to answer question 1 it would be useful to have a clearer understanding of the nature and locus of smooth muscle drug receptors. If such information was available it might be possible to relate the action of cocaine to, for instance, the α-adrenergic

receptor; such knowledge would aid in the answers to questions 2 and 3.

An insight into the changes which cocaine can induce may possibly be obtained by the use of certain physical techniques. Optical rotatory dispersion measurements have been made of biopolymers in contact with drug and substrate molecules (Moore and Wetlaufer, 1971). X-ray diffraction techniques have been used (Perutz, 1964) to demonstrate conformational changes induced by the interaction of haemoglobin and oxygen, and enzymes with enzyme inhibitors (Phillips, 1966). The use of electron spin resonance (ESR) and the spin labeling method in pharmacology have recently been reviewed by Jost and Griffith (1971). Nuclear Magnetic Resonance (NMR) measurements have been used to study the binding of small molecules to proteins and this subject has recently been reviewed by Jardetzky and Wade-Jardetzky (1971). Of related interest to the present work is the finding of Fischer and Jost (1969) where NMR spectral changes associated with the binding of adrenaline to liver cells were noted, these changes were prevented by the β-adrenergic blocking agent dichloroisoproterenol. Metcalfe (1970) has employed both ESR and NMR techniques to the study of the interactions of Ca^{++} and local anesthetics with erythrocyte membranes. The major difficulty in using the NMR technique for pharmacological studies is related to the difficulty in interpretation of the spectrum obtained from biochemically ill-defined parameters. Molecular orbital theory, a technique which can be used to predict the preferred conformation of the drug, has been used by Kier (1971) to obtain an insight into the possible nature of the topology of the receptor.

There are certain techniques available which could be utilized to study the nature of cocaine interaction with the smooth muscle. It would be misleading to assume, however, that such techniques are immediately applicable to this particular problem and clearly interpretation of preliminary results would need to be performed with caution.

V. SUMMARY AND CONCLUSIONS

SUMMARY AND CONCLUSIONS

Several questions were advanced at the beginning of this study and it is useful to briefly summarize the results and conclusions with regard to these four questions.

1. Is the human umbilical artery innervated by adrenergic nerves?

It has been shown that there is no indication of adrenergic nerve fibers in relation to either the two umbilical arteries or the umbilical vein in the extra-abdominal portion of the human umbilical cord.

2. Can noradrenaline be accumulated by human umbilical artery tissue?

Noradrenaline can accumulate in the tissues constituting the umbilical artery, and the preliminary studies show that the tissue to medium ratio seldom exceeded one. The characteristics of noradrenaline accumulation are similar to those seen with extraneuronal uptake of noradrenaline in innervated smooth muscle systems; however, the extraneuronal uptake in this non-innervated tissue is much less sensitive to the inhibitory actions of various drugs.

3. If noradrenaline accumulation can occur in the umbilical artery is it influenced by cocaine?

Noradrenaline accumulation is significantly inhibited by high concentrations of cocaine (3.3×10^{-4}M), but at lower concentrations there is no significant change in the tissue accumulation. The

cocaine concentration which inhibits the "extraneuronal uptake" in the human umbilical artery is several orders of magnitude greater than that observed, by other workers, to have a similar effect on neuronal uptake in innervated tissues.

4. Is the potentiating action of cocaine specific to noradrenaline or can it also influence the action of other smooth muscle agonists?

In the non-adrenergically innervated human umbilical artery preparation responses to noradrenaline, tyramine and 5-hydroxytryptamine are potentiated by cocaine. The action of cocaine, however, has a greater effect on the noradrenaline mediated response. The potentiation by cocaine of the response to noradrenaline is not mediated by blockade of noradrenaline uptake into the effector cell. The responses of two other agonists, histamine and potassium chloride, were not significantly altered by cocaine. Cocaine thus has a selective action on the responses mediated by the α-adrenergic receptor and this action can be explained by an alteration in the reactivity or conformation of the receptor. It was found that at higher cocaine concentrations ($>10^{-4}$M) a non-selective antagonism of contractures of umbilical artery could be demonstrated.

In addition to those which have been answered above, this investigation has suggested numerous other questions. Several recommendations for future research are outlined below:

1. *In vivo* experiments in certain suitable animal preparations would be useful to elucidate the mechanism for closure of the umbilical arteries.

2. A further comparison of the non-innervated umbilical artery preparation with innervated tissues may prove valuable for the study of the roles and functions of extraneuronal noradrenaline uptake. If fluorescence histochemical techniques are employed in such studies it is important that these be accompanied by biochemical measurements of catecholamines.

3. Experiments to elucidate the action of cocaine on smooth muscle physiology would be useful in determining the nature of the postsynaptic action of cocaine. Thus an investigation of the possible interrelationship of cocaine with calcium mechanisms in smooth muscle would be beneficial.

4. Physical techniques would be useful, in conjunction with physiological methods, to elucidate possible cocaine-induced conformational changes.

VI. BIBLIOGRAPHY

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APPENDIX A

PREPARATION OF KREBS SOLUTION:

Three stock solutions were used, from which daily requirements of Krebs solution could be made.

Stock A:

Stock A:	NaCl	76.3 g
	KCl	3.9 g
	$MgCl_2 \cdot 6H_2O$	2.63 g
	$CaCl_2 \cdot 2H_2O$	4.1 g
	$NaH_2PO_4 \cdot H_2O$	1.83 g

in 1000 ml of distilled water

Stock B: $NaHCO_3$ - 2.6%

Stock C: Dextrose - 20%

The solution employed consisted of the following:

Stock A	100 ml
Stock B	80 ml
Stock C	50 ml
Distilled water	1000 ml

This solution had the following composition:

	mM
Na^{++}	128.1
K^{+}	4.3
Ca^{++}	2.27
Mg^{++}	1.1
Cl^{-}	118.04
$H_2PO_4^{-}$	1.1
HCO_3^{-}	20.0
Glucose	45

APPENDIX B

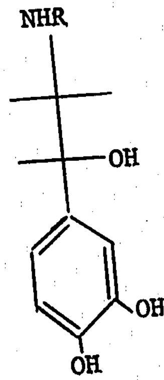
Drug stock solutions, usually 10^{-3}M, were prepared in 0.01N hydrochloric acid and daily supplies were made by appropriate dilution in 0.9% saline.

DRUGS USED:

Cocaine hydrochloride (British Drug House)
Dibenzyline [Phenoxybenzamine sulfate](Smith, Kline and French)
2'[3 dimethyl aminopropyl thio]cinnamanilide hydrochloride [DPTC]
(Nutritional Biochemical Company)
*D(-)Epinephrine bitartrate (Winthrop)
Heparin sodium [1 g = 160,000 U.](Fisher Scientific)
Histamine biphosphate (Nutritional Biochemical Company)
5-hydroxytryptamine [Creatinin phosphate](Nutritional Biochemical Company)
Isopropylnoradrenaline (Winthrop)
*[D(-)]Levarterenol bitartrate (Winthrop)
Oxytocin Synthetic [10 I.U./ml](Nutritional Biochemical Company)
Ouabain octahydrate [Strophanthin G](Sigma Chemicals)
Tranglcyproprine sulfate [Parnate](Smith, Kline and French)
Phentolamine [Regitine]
Propranolol [AY 64043](Ayerst, McKenna and Harrison)
Tyramine hydrochloride (Aldrich Chemical Company)
Vasopressin (Nutritional Biochemical Company)

*Throughout this report, the physiologically-occurring form of noradrenaline (or adrenaline), commonly called 1-noradrenaline, will be referred to by the more accurate designation, D(-) noradrenaline. The D and L symbols are here restricted to describing the absolute configuration of the isomer at the asymmetric carbon, β-carbon (Pratesi, 1963),

whereas the plus (+) and minus (-) signs, representing dextro (right) and levo (left) respectively, indicate the direction in which the isomers rotate plane polarized light. On the basis of the sequence rule (Cahn _et al._, 1956) the more active levorotatory isomers have the R configuration:



APPENDIX C

COMMON ABBREVIATIONS

BOL : Brom-lysergic acid diethylamide

COMT : Catechol-o-methyl transferase

D.A. : ductus arteriosus

DMI : desmethylimipramine

DMPEA: N,N-dimethyl-2-bromo-phenylethylamine

DPTC : 2'[dimethyl aminopropyl thio] cinnamanilide hydrochloride

D.V. : ductus venosus

ED_{50} : effective dose for a 50% response

ESR : electron spin resonance

5HT : 5-hydroxytryptamine

MAO : monoamine oxidase

NMN : normetanephrine

NMR : Nuclear Magnetic Resonance

POB : Phenoxybenzamine

PVP : polyvinyl-pyrrolidone

THI : trihydroxyindole

U.A. : umbilical artery