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CATECHOLAMINE PHYSIOLOGY IN THE SHEEP

A thesis
submitted in partial fulfilment
of the requirements for the Degree
of
Doctor of Philosophy in Biological Sciences
at the
University of Waikato
by
ALLAN JOHN PEARSON

ABSTRACT

A study of catecholamine physiology in the sheep has been carried out with emphasis on the measurement of plasma catecholamine levels and their interactions with lactic acid and cortisol. The plasma dynamics of the catecholamines were first examined under laboratory conditions followed by an assessment of sympathetic responses to a variety of environmental stimuli.

Catecholamine levels were monitored in the plasma of sheep during and after 90 minute infusions of l-dopa, dopamine, noradrenaline and adrenaline. A 2-compartmental pharmacokinetic model of l-dopa plasma dynamics was constructed and the half-lives of the fast and slow decay curves determined. Estimation of the half-lives of dopamine, noradrenaline and adrenaline was prevented by a period of increased endogenous secretion that occurred on the termination of the infusions. A hypothesis based on the hypotension developed by sheep on the arrest of catecholamine infusions of this nature was developed to explain the adrenergic activity observed. Lactic acid levels rose markedly during the adrenaline infusions with a much smaller response during noradrenaline infusions while a biphasic cortisol response to both the noradrenaline and adrenaline infusions was observed.

A set of blood samples was collected from each of 6 cannulated lambs at various stages of a journey from the farm to the abattoir. Catecholamine levels remained consistently elevated throughout the experiment before rising to a very high peak upon electrical stunning prior to slaughter. Plasma lactic acid peaks were associated with mustering and washing while cortisol remained high throughout the experiment until penning at the abattoir when it fell sharply to baseline levels. Subsequently it rose overnight. The physiological basis of these observations is discussed in relation to the transport of stock and the use of these blood substances in assessing the reactions of domestic animals to their environment.

The massive catecholamine release in response to electrical stunning was further studied in a series of experiments. Significant differences between lamb mobs slaughtered at an abattoir appeared but a relationship was not found between the catecholamine plasma concentrations and the haemorrhagic lesion known as "blood splash" caused by electrical stunning. Short-term stressors encountered by the lambs at the abattoir had no effect on the blood splash incidence but some evidence suggested a link with the previous behavioural and handling conditions of slaughtered lambs. Both the α -adrenergic and the β -adrenergic blocking drugs phentolamine and propranolol were effective in suppressing blood splash and the physiological basis of this observation is considered.

Some preliminary experiments to investigate catecholamine involvement in the neurological disease of ryegrass staggers in sheep are described together with a description of the intense ingestive behaviour and the possible taming effects induced by the central dopaminergic receptor blocker haloperidol.

The application of these and other results to future agricultural research programmes is considered in the light of recent developments in the understanding of the multiple roles of these substances in mammalian physiology.

ACKNOWLEDGMENTS

I would especially like to thank my supervisors Dr W.G. Whittlestone and Dr M.A. Chapman for their constant enthusiasm and encouragement and for their constructive suggestions during this study. My technician Jill Braggins has been of essential assistance in all the experiments described and her willing efforts are truly valued. The cheerful assistance of Ian Potts in the latter stages of the data analysis has also been much appreciated.

Dr Ron Kilgour and Kaye Bremner have generously given much time in stimulating discussion and in assisting with experiments where aspects of animal behaviour have been involved. Hank de Langen is thanked for his willingness to lend a hand whenever the need arose, for his careful art work and advice on technical matters. Special acknowledgements are due to Stan Willson for his detailed critical review of the manuscript in its early stages.

I would also extend my thanks and appreciation to the following -

David Duganzich for his work and guidance in the statistical analysis of the experimental results and Henry Bishop for his help in the surgery.

The staff of the Fertility Centre and the No 3 Dairy for their patience and co-operation during various experiments.

Gary Leet for the electron microscopy and the production of the electron micrographs and Dr Bob Fairclough for the cortisol analyses.

The typists in the typing pool, the glass blower, the photographer, the computer operators and the administrative staff of the Ruakura Agricultural Research Centre whose help has been essential.

Marion France for her skill and patience in the typing of this thesis.

Dr Alan Kirton, members of the Meat Industry Research Institute of New Zealand and the men of the Ruakura and the Horotiu abattoirs for their contributions to the experiments relating to the transport and electrical stunning of slaughter stock.

The Ruakura library staff, particularly Alice Buckle, Mary Ryan and Leslie Stannard for their response to the heavy demands made of them.

Finally I would like to acknowledge the essential role of the Ministry of Agriculture and Fisheries in granting me the study leave and the use of their facilities and resources to carry out the work described in this thesis. The personal interest and kindness of Barrie Keenan in supervising my study leave have been much appreciated.

To all these people and to my other friends and colleagues who contributed in very many ways to the progress of this work I express my deep gratitude.

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LIST OF ABBREVIATIONS

A	adrenaline
ACTH	adrenocorticophic hormone
ADP	adenosine 5'-diphosphate
amp	ampere
AR	analytical reagent
ATP	adenosine 5'-triphosphate
CA	catecholamine
CG	commercial grade
Ci	Curie
CNS	central nervous system
COMT	catechol-O-methyltransferase
cpm	counts minute ⁻¹
CRH	corticotrophin releasing hormone
cyclic AMP	cyclic adenosine 3', 5'-monophosphate
DA	dopamine
D β H	dopamine β -hydroxylase
DDC	dopa decarboxylase
DHPE	3,4-dihydroxyphenylethanol
DHPG	3,4-dihydroxyphenylglycol
dl	decilitre
DOMA	3,4-dihydroxymandelic acid
DOPAC	3,4-dihydroxyphenylacetic acid
dpm	disintegrations minute ⁻¹
EC	enzyme commission
EDA	ethylenediamine
EDTA	ethylenediamine tetraacetic acid
g	gravity
GLC	gas-liquid chromatography
ha	hectare
Hgb	haemoglobin
hr	hour
Hz	hertz
HVA	homovanillic acid
id	inside diameter
ip	intraperitoneal
IU	international unit
iv	intravenous
kg	kilogram
km	kilometre
l	litre
LDH	lactic dehydrogenase
LR	laboratory reagent
m	metre
MAO	monoamine oxidase
mg	milligram
MHPE	3-methoxy-4-hydroxyphenylethanol
MHPG	3-methoxy-4-hydroxyphenylglycol
min	minute
ml	millilitre
mm	millimetre

mm Hg	millimetres of mercury
mmole	millimole
MN	metanephrine
MT	3-methoxytyramine
MW	molecular weight
n	number
NA	noradrenaline
NAD	nicotinamide-adenine dinucleotide
NADH	reduced NAD
ng	nanogram
NMN	normetanephrine
ns	non-significant
od	outside diameter
PVC	packed cell volume
pg	picogram
PHE	phenylalanine
PNMT	phenylethanolamine N-methyltransferase
POPOP	1,4-bis-2-(5-phenyloxazolyl)-benzene
PPO	2,5-diphenyloxazole
RBC	red blood cell
SAM	S-adenosylmethionine
SC	subcutaneous
SD	standard deviation
SE	standard error of the mean
SG	scintillation grade
sp gr	specific gravity
TH	tyrosine hydroxylase
THI	trihydroxyindole
TLC	thin layer chromatography
TYR	tyrosine
uv	ultraviolet
v	volume
VA	vanillic acid
VMA	vanilmandelic acid
w	weight
WBC	white blood cell
μg	microgram
μm	micrometre
°	degrees Celcius
§	section

INTRODUCTION

The biogenic amines reach into every area of animal physiology as hormones and neurotransmitters relaying and integrating the stimuli generated by physiological transducers in response to both external and internal events. Over the last 30 years there has occurred an exponential course of progress in the understanding of these ubiquitous compounds, and in particular the group known as the catecholamines. Increasingly it has become apparent that the results of the inter-disciplinary study of the catecholamines are directly applicable in many research programmes aimed at increasing animal productivity, whether these be lactation, reproduction, meat quality, behaviour or animal health. Yet to date primates and small laboratory animals have been almost exclusively the subjects of catecholamine research with little attention given to the domestic animal. The work presented in this study developed from a wish to study the catecholamines in the context of current agricultural research. As only a limited area could be studied in detail an additional objective was to consider the present work in the widest possible terms, to gather information and acquire skills applicable to other existing or potential agricultural problems.

A general review of the catecholamines, their functions, biochemistry release and assay, is first presented in Chapters 1-3. A necessary prerequisite of the proposed research programme was the development of assays sufficiently sensitive and precise to determine blood-borne catecholamines (Chapter 4). The physiological consequences of catecholamine infusions in sheep are examined in Chapter 5 and the results extended to the physiological responses of sheep to transport (Chapter 6), electrical stunning and slaughter (Chapter 7). The possible involvement of catecholamines in ryegrass staggers is covered in Chapter 8 together with the behavioural effects of a central nervous system catecholaminergic receptor blocker.

Simultaneously with the measurement of the catecholamines, plasma cortisol and lactic acid were also determined in most experiments. These substances, lactic acid (a product of carbohydrate metabolism and a determinant of the ultimate quality of meat) and cortisol (a measure of the perceived reaction to external stimuli and with wide ranging metabolic effects in its own right) have contributed to the interpretation of the experimental results. Just as important however are the insights gained into the interactions of the catecholamines with other blood components.

Throughout this thesis the symbol " $\pm$ " is used to introduce the estimated standard deviation (SD) of a population. Where the standard error (SE) of the mean is employed it is always identified as such. The units of measurement for the plasma catecholamines, cortisol and lactic acid are expressed in terms of ng ml^{-1} , ng ml^{-1} and mg dl^{-1} respectively to facilitate comparison with the bulk of the literature results relating to these substances.

THE CATECHOLAMINES :

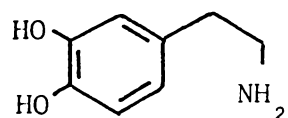
GENERAL INTRODUCTION

CHAPTER 1

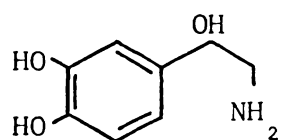
1.1 INTRODUCTION

The principal mammalian catecholamines are dopamine (DA), noradrenaline (NA) and adrenaline (A); distinguished by their possession of a 3,4-dihydroxyphenyl (catechol) nucleus (Figure 1-1). The diversity and wide distribution of the catecholamines is reflected in the variety of functions these compounds serve - including aspects of animal metabolism, numerous physiological adaptations, nervous transmission, behaviour, and hormone release.

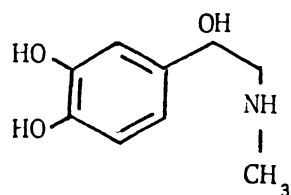
Until comparatively recently dopamine was thought of only as an intermediate in the synthesis of noradrenaline and adrenaline (§2.2) but is now recognized to have important effects in its role as a neurotransmitter in the central nervous system (CNS) (Fuxe & Hökfelt 1969, Vogt 1973, Krnjević 1974). Dopaminergic neurones are also found in sympathetic ganglia (Björklund *et al.* 1970) and evidence is mounting for a variety of peripheral functions (Thorner 1975). The cardiovascular and renal effects of dopamine differ in important respects from other sympathomimetic amines (McDonald *et al.* 1964, Goldberg 1972, Hollenberg *et al.* 1973), though these are of an uncertain physiological significance. While noradrenaline is secreted by the adrenal medulla and has physiological and metabolic effects distinct from adrenaline (Himms-Hagen 1967, Koelle 1970), it is primarily located in postganglionic sympathetic neurones and in certain areas of the central nervous system serving as a synaptic transmitter (Krnjević 1974). Adrenaline is a classical hormone; synthesized and secreted by the adrenal medulla, transported to the target tissues in the bloodstream and taken up by specific receptors with widespread physiological and metabolic effects (Himms-Hagen



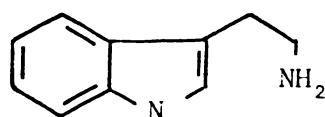
dopamine



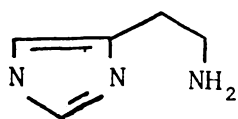
noradrenaline



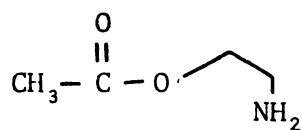
adrenaline



serotonin



histamine



acetylcholine

Figure 1-1. Structure of the biogenic amines.

1967, Koelle 1970). Adrenaline is the hormone through which the adrenal medulla exerts its metabolic and circulatory control under conditions of stress. It is also present in small quantities in the central nervous system (von Euler 1946, Gunne 1962, Koslow & Schlumpf 1974) though with, as yet, an undefined function. The catecholamines are important in the functioning of the hypothalamus, the adrenal medulla and the pineal gland, all of which can be considered neuro-endocrine interfaces and they also illustrate the close interrelationship between the nervous and endocrine systems in the transition from the predominantly nervous role of dopamine, through noradrenaline to the predominantly endocrine role of adrenaline.

1.2 HISTORICAL DEVELOPMENT OF CATECHOLAMINE RESEARCH

Though the existence of the adrenals and the concept of nervous transmission were well known in the nineteenth century, modern research on the physiology of the catecholamines began about 1900. A biologically active pressor substance in adrenal medulla extracts was described by Oliver & Schäfer (1895) and Cybulski (1895). Cybulski further showed the presence of the active substance in the adrenal venous blood under conditions of stress and that the pressor activity of the urine was increased by the intravenous injection of adrenal medulla extracts. The pressor was soon extracted and purified (Abel 1899), identified as N-methyl-3,4-dihydroxyphenyl-ethanolamine (Takamine 1901) and synthesized along with its primary amino homologue, noradrenaline (Stolz 1904).

Struck by the similarity in the physiological effects of administered adrenaline and sympathetic nerve stimulation, Elliot (1904, 1905) suggested that sympathetic nerve impulses released minute quantities of an adrenaline-like substance at nerve endings. From this hypothesis arose the modern concept of chemical synaptic transmission. Dixon (1906) and Dixon & Hamill (1909) extended the theory by analogy to muscarine and vagal transmission. However it was not until 1921 that Otto Loewi showed that stimulation of the vagus nerve to an isolated frog heart liberated a substance which slowed a second heart and, furthermore, that stimulation of the cardiac sympathetic fibres released a substance which accelerated

another heart. While the inhibitory substance was shown to be acetylcholine (Lowei & Navratil 1926) and the theory of chemical transmission gained acceptance, the identity of the sympathetic transmitter remained obscure; even up to 1946 it was often assumed that it was adrenaline. While most physical and biological tests available in 1910 indicated that the transmitter was adrenaline, Barger & Dale (1910) questioned this assumption on the basis of small, but significant, discrepancies in the action of adrenaline and those brought about by nervous stimulation. Cannon & Uridil (1921) were able to demonstrate that the remote effects of the substance produced by the stimulation of splanchnic or hepatic nerve *in vivo* were not identical with those produced by adrenaline. In the absence of direct chemical identification Cannon and his co-workers termed the transmitter *sympathin*. Later authors (Melville 1937, Stehle & Ellsworth 1937, Greer *et al.* 1938) noted similar discrepancies. The facts suggested to these physiologists that adrenaline and the sympathetic transmitter were different and indeed that there were two types of sympathin (Cannon & Rosenblueth 1933) - one excitatory to muscle cells (sympathin E) and one inhibitory (sympathin I). This latter and, at the time, confusing observation is now accounted for by the postulated existence of two types of receptors (termed α and β receptors) on many cells. These receptors have differing sensitivities to various agonists and antagonists (Nickerson 1959, Ahlquist 1962, Jenkinson 1973). Eventually after the extraction of the transmitter from sympathetic nerves it was identified as noradrenaline (von Euler 1946, von Euler 1948). The first observation directly showing the release of noradrenaline by stimulated sympathetic nerves was made by Peart in 1949. During this period Cannon also established adrenal medullary adrenaline release as a primary reaction to stress and developed an awareness of the functional unity of the sympathetic-adrenal system in response to emergencies (Cannon 1928, Cannon 1931).

The third important catecholamine, dopamine, was recognized by Holtz *et al.* (1942) as a naturally occurring substance in human urine. It was Goodall (1950, 1951) who discovered dopamine in the adrenal medulla; in the sympathetic nerves it was first found by Schümann (1956a,b). The possibility of dopamine being a

synaptic transmitter was taken up as soon as its place in the synthetic pathway of adrenaline was established. In 1954 Vogt showed that catecholamines occurred in the brain; that nor-adrenaline was the predominant catecholamine and that it occurred chiefly in the hypothalamus and the brain stem. Dopamine was soon also found in the central nervous system (Montagu 1957, Weil-Malherbe & Bone 1957a) and shown to be present mainly in the basal ganglia unaccompanied by noradrenaline (Bertler & Rosengren 1959).

The development of a sensitive fluorescence histochemical technique for the direct identification of catecholamine-containing structures (Falck *et al.* 1962) has allowed the detailed mapping of dopamine and noradrenaline pathways in the central nervous system (§1.3). The ensuing research into the roles of the catecholamine-containing neurones of the central nervous system is still in its infancy (Vogt 1973).

Present understanding of catecholamine biochemistry dates from 1939 with the discovery of l-dopa decarboxylase (Holtz 1939) and the proposed, later to be confirmed, biosynthetic pathway of adrenaline from tyrosine (Blaschko 1939). With all the enzymes involved in this transformation now characterized, attention has shifted to the intricate mechanisms controlling the biosynthesis and metabolism of the catecholamines (§2.3). An interesting aspect of the biochemical study of the catecholamines was the discovery that the metabolic actions of adrenaline are mediated through cyclic adenosine 3',5'-monophosphate (cyclic AMP), establishing the idea of a *second hormonal messenger*, now extended to numerous hormones (Sutherland & Rall 1960).

1.3 CATECHOLAMINE DISTRIBUTION

The catecholamines are distributed throughout the animal kingdom. They have been found in micro-organisms (Janakidevi *et al.* 1966), in invertebrates (Östlund 1954, Sekeris & Karlson 1966), in birds (Ghosh 1962, Juorio & Vogt 1967) and in mammals (Höckfelt 1951). Their occurrence in plants has been noted (Waalkes *et al.* 1958,

Udenfriend *et al.* 1959). The relative importance of individual catecholamines shows a marked variation from one group to another (von Euler 1963). In contrast to mammals for example, dopamine is the predominant catecholamine in the great majority of invertebrate phyla, noradrenaline is generally present to a much lesser extent and adrenaline is almost completely absent (Welsh 1972).

In mammals dopamine, noradrenaline and adrenaline are found throughout the body, notably associated with chromaffin cells and nervous tissue. The most characteristic feature of the chromaffin cell, originally named for its distinctive staining properties with chromic compounds, is the presence of large numbers of membrane-bound osmophilic granules which vary in size from 50-400 nm. These represent catecholamine-containing structures and may be isolated by cell homogenization and centrifugation (Hillarp & Nilson 1954). From an embryological viewpoint the sympathetic chains are generally believed to derive from the neural crest of the ectoderm (Boyd 1960). Some of these fibres reach the neighbourhood of the primordium of the adrenal cortex accompanied by chromaffinoblasts which differentiate into the chromaffin cells of the adrenal medulla. Thus both chromaffin and sympathetic elements are closely associated during development and both arise from the small, intensely basophilic cell designated as a *primitive sympathetic cell* (Coupland 1965a, Polak *et al.* 1970). This may explain why chromaffin cells are found in a variety of organs supplied by sympathetic nerves (Coupland 1965a). The use of the term chromaffin cell with respect to catecholamine-containing cells outside the adrenal medulla is confused but according to Jacobowitz (1970) it signifies *those cells that resemble the chromaffin cells of the adrenal medulla in shape and size, contain either noradrenaline or adrenaline, are not mast cells or enterochromaffin cells, and show brownish stains with bichromate solutions.*

Small amounts of mainly noradrenaline are found in extramedullary chromaffin cells. These are singly, or in small aggregates, scattered throughout the body including, for example, the urethral mucosa (Dixon *et al.* 1973) and the carotid body (Lever *et al.* 1959). Clusters of chromaffin cells have also been observed in

sympathetic and cholinergic nervous tracts and ganglia (Eränkö & Härkönen 1963, Jacobowitz & Woodward 1968, Costa & Furness 1973, Jacobowitz *et al.* 1973). Jacobowitz (1970) suggested that these cells exert a modulatory influence on impulse transmission through the ganglia.

In the adult mammal the adrenal medulla represents the bulk of the adrenaline-containing chromaffin cells. Holzbauer & Sharman (1972) reported a total catecholamine concentration of 0.12-2.23 mg g⁻¹ in medullary tissue of various mammalian species. The adrenal medulla also contains significant quantities of noradrenaline (Holtz *et al.* 1947). Not all the noradrenaline represents adrenaline precursor since, within the adrenal medulla, the two amines occupy specific cells, differentially distributed (Palkama 1964) and with different staining properties (Coupland 1971). The noradrenaline-containing granules are polymorphous and extremely electron-dense while the adrenaline-storing granules are round and show a lesser electron-density (Piezzi & Cavicchia 1973) (§7.2). While mixtures of noradrenaline and adrenaline-storing granules occur during foetal life and for a few days afterwards, it appears that thereafter individual chromaffin cells store only one form of the amine.

Large variations in the relative proportions of medullary noradrenaline and adrenaline occur between different mammalian species; in general rodents have the lowest percentage of noradrenaline while cats and lions have the highest (von Euler 1963). A considerable range in the noradrenaline/adrenaline ratio is also seen within a species at birth, but in almost all cases there is an increase in the proportion of adrenaline with age until the adult values are reached (Shepherd & West 1951). In this connection it should be noted that during the foetal period the bulk of the chromaffin tissue lies outside the adrenal medulla, certainly in the case of rabbits and humans (Coupland 1965a). The largest discrete chromaffin bodies form paired or fused collections of cells adjacent to the origin of the inferior mesenteric artery and are intimately associated with the preaortic nerve plexus in this region. These are the organs of Zuckerkandl (Zuckerkandl 1901, 1912). In early

postnatal life the organs of Zuckerkandl and other extra-adrenal chromaffin tissue degenerate to a large extent while the adrenal medulla increases markedly in size (West *et al.* 1953).

Dopamine is present in the adrenal medulla but in a much lower concentration than is found for adrenaline (Vandermeulen *et al.* 1968, Cession-Fossion & Vandermeulen 1969). There is some evidence for the existence of discrete dopamine-containing granules (Lishajko 1968). In certain tissues such as bovine lung (von Euler & Lishajko 1957), duodenal mucosa and submucosa (Bertler *et al.* 1959, Coupland & Heath 1961), dopamine is found in large quantities stored in chromaffin-like cells. The structural characteristics of these cells are different from the noradrenaline and adrenaline-storing cells and they are believed to be a special form of chromaffin cell (Bertler *et al.* 1959). Coupland & Heath (1961), however, identified them as mast cells. Dopamine is disseminated throughout noradrenergic structures (Schümann 1958, Costa *et al.* 1972) - presumably as noradrenaline precursor (§2.2).

All organs supplied by sympathetic noradrenergic nerves contain noradrenaline in amounts directly proportional to the extent of such innervation (von Euler 1956) (Table 1-1).

Table 1-1
Noradrenaline Content of Organs Supplied
with Adrenergic Nerves ($\mu\text{g g}^{-1}$ fresh tissue)

Organ	Cow	Sheep	Cat	Rat
Heart	0.3 - 0.6	0.6 -1.1	0.5 -1.0	0.5-0.65
Liver	0.25	0.2 -0.5	0.05-0.2	0.4-0.6
Spleen	1.5 - 3.5	1.6 -3.3	0.8 -1.4	0.4-0.5
Kidney	0.04- 0.1	0.4 -0.6	0.1 -0.3	-
Blood vessels	0.3 - 0.5	-	-	-
Lung	0.05	0.1	-	-
Skeletal muscle	0.04	0.03-0.07	0.03	0.05
Splenic nerves	8.5 -18.5	-	-	-

[from von Euler 1963]

Since preganglionic denervation leads to an almost complete disappearance of noradrenaline from most tissues and since the noradrenaline content increases progressively with nerve regeneration it is thought that the noradrenaline is almost wholly contained within the sympathetic nerve fibre (von Euler & Purkhold 1951). Dopaminergic neurones have been reported in sympathetic ganglia (Björklund *et al.* 1970).

Usually the adrenaline content of tissue extracts is small, constituting 2-10% of the total catecholamine content (von Euler 1956). After section and degeneration of postsynaptic adrenergic nerves the noradrenaline almost completely disappears but a large proportion of the adrenaline remains, perhaps within chromaffin cells (von Euler & Purkhold 1951). Whether this represents adrenaline actively biosynthesized by the cells or originates from the uptake of circulating adrenaline (Strömblad & Nickerson 1961) is unknown.

Both noradrenaline (Vogt 1954, Vogt 1957) and dopamine (Montagu 1957, Weil-Malherbe & Bone 1957a, Bertler & Rosengren 1959, Carlsson 1959) are present in significant quantities in the central nervous system. Fluorescence histochemistry, supported by data obtained through the use of surgical, biochemical, pharmacological and autoradiographic techniques, has resulted in the tracing of the catecholamine-containing pathways in the brain (Ungerstedt 1971, Livett 1973). The formaldehyde fluorescence technique is based on the fact, as originally shown by Eränkö (1955) that formaldehyde and monoamines react to give fluorescent yellow-green isoquinolines. Thus it has been shown that the catecholamines are concentrated in specific areas, that the individual catecholamines are present in specific neurones, and that they are present in highest concentration in their axonal processes, thereby suggesting a neurotransmitter role for the catecholamines. Allowing for the fact that dopamine-containing neurones take up noradrenaline, Reivich & Glowinski (1967) found a good correlation between those areas labelled by ^{14}C -noradrenaline and the catecholamine-containing neurones mapped by other techniques.

Strikingly high concentrations of dopamine occur in the neostriatum (Figure 1-2a). The amount of noradrenaline in the central nervous system is relatively small (ranging overall from 0.2 to 0.5 $\mu\text{g g}^{-1}$), though widely distributed. Concentrations in the hypothalamus and brain stem are noticeably higher than those in other regions (Figure 1-2b). While the absolute concentration of noradrenaline in any particular brain region may differ slightly among different mammalian species, the percentage of total brain noradrenaline present in any given region is relatively constant from one species to another (Dahlström 1971b). The regional distribution of both dopamine and noradrenaline is correlated well with the distribution of their biosynthetic enzymes (Hartman *et al.* 1972, Hökfelt *et al.* 1973a), as determined by immunohistochemistry (Hökfelt *et al.* 1973b). Small quantities of adrenaline (von Euler 1946, Gunne 1962, Koslow & Schlumpf 1974) and the enzyme responsible for its formation from noradrenaline (phenylethanolamine N-methyl transferase) (Saavedra *et al.* 1974) have been found in the brain raising the possibility that adrenaline itself may be a neurotransmitter.

The functions of the central catecholamine neurones appear to be in the modulation and fine control of various vascular, endocrine, and neuronal systems ensuring homeostasis. However most of the numerous roles postulated for central nervous system catecholamines (Vogt 1973, Krnjević 1974) lack definitive supporting evidence.

1.4 STRUCTURE OF THE SYMPATHETIC NEURONE

From light and electron microscopic studies there is general agreement that the adrenergic innervation of the glands and smooth and cardiac muscle consists of a network of very fine varicose nerve fibres arising from the sympathetic axon (Figure 1-3). The fibres are enclosed in a plexus of anastomosing strands of Schwann cells. The extensive branching observed within the network and the very considerable length of the fibres results in a high degree of tissue innervation (Richardson 1962). Silver stained nerve fibres in this plexus have a beaded appearance

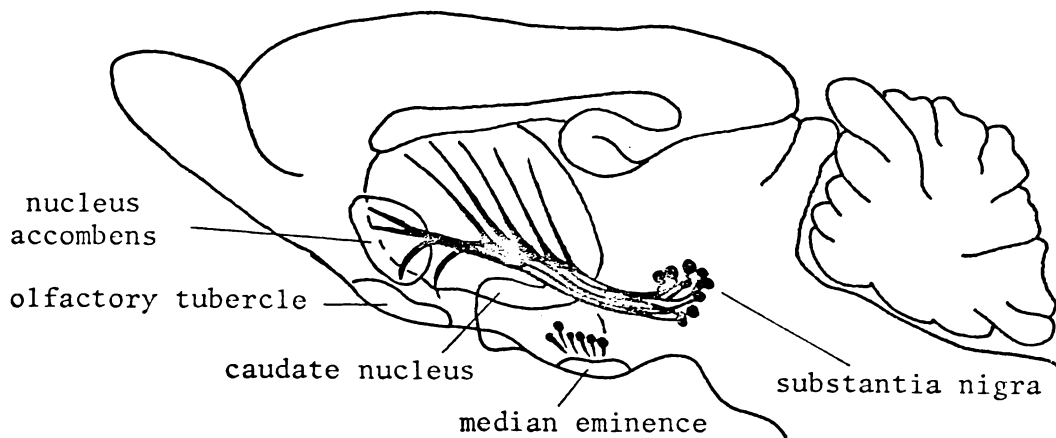


Figure 1-2a. Dopamine tracts in a longitudinal section along the midline of the rat brain. The cell bodies are concentrated in the substantia nigra.

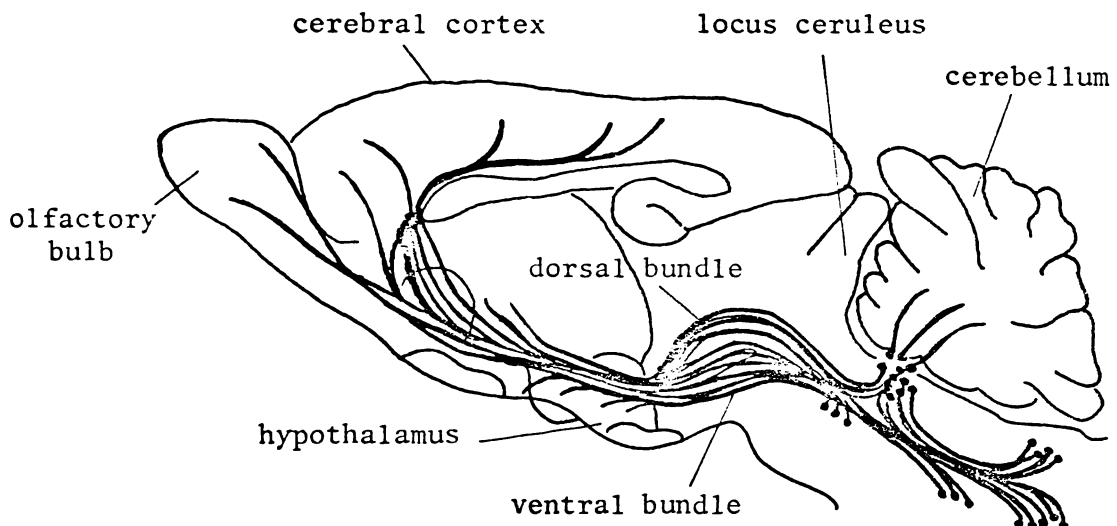


Figure 1-2b. Noradrenaline tracts in a longitudinal section along the midline of the rat brain. The noradrenaline neurones arise mainly in the locus ceruleus and reach many brain centres.

[from Axelrod 1974]

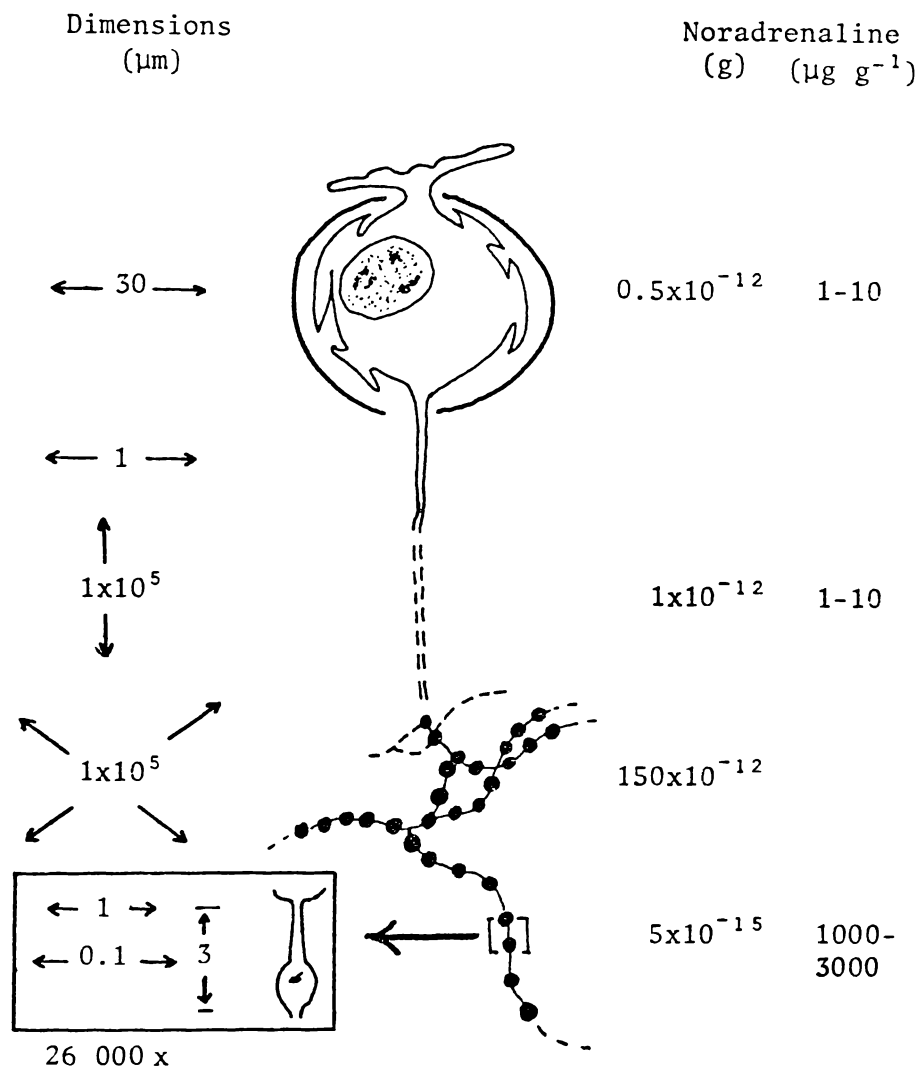


Figure 1-3. Dimensions of a sympathetic neurone (cat superior cervical ganglion) and the distribution of noradrenaline. [from Geffen & Livett 1971]

(Garven & Gairns 1952), and fluorescent specimens show a corresponding series of bright varicosities (Falck 1962, Norberg & Hamberger 1964).

The axon may extend up to 100 mm or more before branching into its terminal network. The beaded varicosities are about 1 μm in diameter, the intervaricosities about 0.1 μm with successive varicosities occurring approximately every 3 μm (Geffen & Livett 1971).

It is now well established from fluorescence microscopy that noradrenaline is unevenly distributed within the neurone with high concentrations in the nerve endings, low concentrations in the axon and somewhat variable concentrations in the cell body (Dahlström 1971a). Within the axon virtually all the noradrenaline is located within specialised subcellular particles, the dense-cored granules or vesicles (von Euler & Hillarp 1956). As the nerve ending is approached the proportion of noradrenaline that is granule-bound increases. In homogenates of splenic nerve trunks only 30% of the noradrenaline sediments with the granular fraction (von Euler & Lishajko 1961a) while in cardiac homogenates, where most of the sympathetic nervous tissue is present as nerve endings, the bound noradrenaline approaches 100% of the total (Potter & Axelrod 1963). Electron microscopic studies (de Robertis & Pellegrino de Iraldi 1961, Grillo & Palay 1962, Tranzer & Thoenen 1968) have revealed the presence of at least two types of dense-cored vesicles with a diameter of 30-60 nm (dense core diameter 15-25 nm) and 60-150 nm (core diameter 40-70 nm) respectively. A third type of vesicle in axon varicosities is the small agranular vesicle with a diameter of 30-60 nm, morphologically indistinguishable from the synaptic vesicles seen in most cholinergic nerve endings (Grillo & Palay 1962). Following osmium fixation the small granular and agranular vesicles are found in approximately equal proportions (Richardson 1962).

Similar larger storage organelles are observed within chromaffin cells (Blaschko & Welch 1953). Chromaffin cell granules range in size from 50-400 nm (Lever *et al.* 1959), while granules of 40-60 nm and 80-130 nm exist within the central nervous system catecholamine-containing neurones (Pellegrino de Iraldi *et al.* 1963, Hökfelt 1967, Bloom & Aghajanian 1968).

Extensive autoradiographic (Wolfe *et al.* 1962), pharmacological and physiological (Bondareff & Gordon 1966, Van Orden *et al.* 1966, Tranzer & Thoenen 1967, Van Orden *et al.* 1967a) evidence relates the small granular vesicles in sympathetic nerves to the monoamine content. There is also good correlation between the intraneuronal distribution of noradrenaline as revealed with fluorescence microscopy and the small granular vesicles after potassium permanganate fixation (Hökfelt 1969, Van Orden *et al.* 1970). The loss of noradrenaline in transplanted dog hearts (Potter *et al.* 1965) and in the denervated nictitating membranes of the cat (Van Orden *et al.* 1967b) accompanies the disappearance of the small granular vesicles from the nerves. These and other studies strongly indicate that the granular material of the vesicle either in whole or more likely in part, represents the noradrenaline.

The large granular vesicles constitute less than 5% of the vesicle population (Folkow *et al.* 1967, Farrell 1968). The fact that they are resistant to reserpine, a catecholamine-depleting drug, has been taken as evidence that they do not store noradrenaline. However there is some evidence to the contrary and this question and the question of the function of the large granular vesicles remain unanswered (Geffen & Livett 1971).

The distribution of amines and storage vesicles is markedly changed when axons are ligated, with the rapid accumulation of catecholamines above the lesion (Dahlström 1965). Some amine synthesizing enzymes (Dahlström & Jonason 1968, Laduron & Belpaire 1968a) as well as granular vesicles (Kapeller & Mayor 1967) also accumulate above a ligation. It is thought that the various intraneuronal components are transported by axoplasmic streaming from the cell body through the axon to the terminals

(Dahlström 1971a). However, whereas the macromolecular and subcellular particles such as enzymes and storage vesicles (or their precursors) probably have to be synthesized in the cell bodies the transmitter itself can be synthesized along the neurone. The vast majority of noradrenaline molecules released at the synapses are probably synthesized in the nerve endings. Hökfelt (1973) has presented evidence for the local elaboration of storage vesicles in the nerve terminals after reserpine treatment.

Nevertheless the possibility also exists that noradrenaline is stored in non-vesicular locations such as the smooth endoplasmic reticulum, especially in the cell body but also in nerve endings (Tranzer 1972).

THE CATECHOLAMINES: BIOSYNTHESIS, METABOLISM, RELEASE, AND UPTAKE

CHAPTER 2

2.1 INTRODUCTION

The biosynthesis and metabolism of the catecholamines are now comparatively well understood; less is known about the control of these processes and the storage and release of catecholamines. From a pharmacological point of view catecholamine biochemistry is particularly important since many drugs inhibit one or more of the enzymes involved. Alterations in catecholamine biosynthesis, metabolism and storage are implicated in many aspects of brain function (Kety 1966, Schildkraut *et al.* 1967, Charalampous & Brown 1967, Musacchio *et al.* 1969). The mode of catecholamine storage and release is of interest to physiologists studying the mechanisms of synaptic transmission. The chemical structures of the catecholamines and their principal metabolites may be found in Appendix I.

2.2 CATECHOLAMINE BIOSYNTHESIS

In 1906 Hallé first suggested, on the basis of the similarity in chemical structures, that the catecholamines might be derived from either phenylalanine or tyrosine. The first real evidence for a biosynthetic pathway from 3,4-dihydroxyphenylalanine (l-dopa) to the catecholamines was supplied by Imaizumi in 1938, followed by the discovery of the enzyme responsible for the formation of l-dopa from tyrosine (Holtz 1939). On the basis of this and other evidence Blaschko (1939) suggested the now accepted series

of biochemical transformations from tyrosine to l-dopa to dopamine to noradrenaline and finally to adrenaline (Figure 2-1). Gurin & Delluva (1947) and Udenfriend *et al.* (1953) demonstrated that labelled phenylalanine administered to rats was incorporated into adrenaline by the adrenal medulla. The existence of the various intermediates in the process were also confirmed isotopically (Masuoka *et al.* 1956, Rosenfeld *et al.* 1958). With the isolation of tyrosine hydroxylase in 1964 (Nagatsu *et al.* 1964a,b) all the individual enzymes involved in the transformations have now been isolated and characterized in varying degrees of detail.

While tyrosine is the major catecholamine precursor, phenylalanine can be converted to tyrosine by phenylalanine hydroxylase in the liver (Cartwright *et al.* 1973). Tyrosine hydroxylase, which catalyses the conversion of tyrosine to l-dopa requires a reduced pteridine as a cofactor (Nagatsu *et al.* 1964a,b), and is also capable of hydroxylating phenylalanine to tyrosine (Ikeda *et al.* 1965). Theoretically therefore, it is possible for the conversion of phenylalanine to the catecholamines to occur in the adrenal medulla, brain and in the various sympathetically innervated tissues. However, since tyrosine is normally present in the circulation at levels of 10-15 $\mu\text{g ml}^{-1}$ (Spector *et al.* 1963) and is taken up (at least in the brain but presumably elsewhere) by an active transport mechanism (Chirigos *et al.* 1960) it is thought that phenylalanine is not a significant precursor. Dopa decarboxylase which catalyses the next step in the reaction sequence (Holtz *et al.* 1938, Holtz 1939) requires pyridoxal phosphate as a cofactor, and is capable of decarboxylating a number of aromatic l-amino acids. The lack of substrate specificity has led to the use of an alternative name-aromatic l-amino acid decarboxylase (Lovenberg *et al.* 1962, Udenfriend 1962). The side chain hydroxylation of dopamine to noradrenaline is catalysed by dopamine β -hydroxylase. The enzyme contains copper and requires ascorbic acid as a cofactor (Friedman & Kaufman 1965a, Friedman & Kaufman 1965b, Goldstein *et al.* 1965). The final step in the conversion of tyrosine to adrenaline is carried out by phenylethanolamine N-methyltransferase which uses the methyl group of

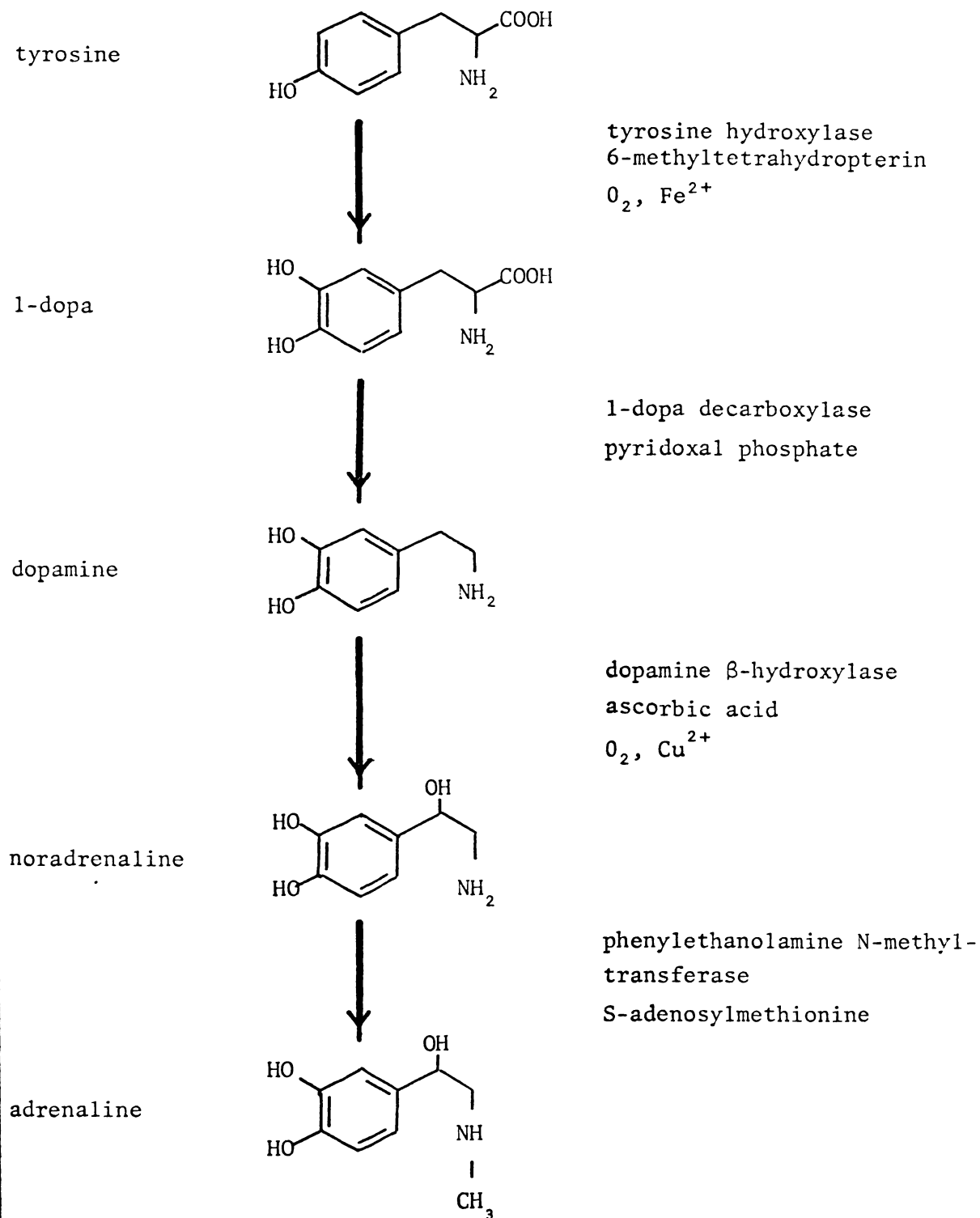


Figure 2-1. Catecholamine biosynthetic pathway.

S-adenosylmethionine to N-methylate noradrenaline and (*in vitro*) a variety of other phenylethanolamine derivatives (Kirshner & Goodall 1957a, Axelrod 1962). Each type of catecholamine-synthesizing cell contains only those biosynthetic enzymes that are active in the elaboration of the chemical messenger that is released. In the following sections each of these enzymes will be described in more detail. The enzyme commission (EC) number given for each enzyme is from the Enzyme Nomenclature Recommendations (1972) of the International Union of Biochemistry.

Tyrosine Hydroxylase (TH) (EC 1.14.16.2)

Tyrosine hydroxylase is an oxygenase which catalyses the conversion of l-tyrosine to l-dopa, requiring a reduced tetrahydropteridine cofactor and oxygen for activity. Iron (Fe^{2+}) also appears to be a cofactor (Nagatsu *et al.* 1964a, Nagatsu *et al.* 1964b, Ikeda *et al.* 1966, Poillon 1973). The reduced pteridine combines with the inactive, oxidized form of the enzyme, converting the latter to the active, reduced form. In turn the pteridine is oxidized and must then be enzymatically reduced before it can again function to regenerate the active form of the enzyme (Ikeda *et al.* 1966). The natural cofactor of tyrosine hydroxylase has only recently been discovered (Lloyd & Weiner 1971, Nagatsu *et al.* 1972); it is 6-methyltetrahydropterin.

The following organs are reported to have enzyme activity (coinciding with the tissue distribution of catecholamines): adrenal medulla, brain, spleen, heart (Nagatsu *et al.* 1964b), salivary glands (Sedvall & Kopin 1967), splenic nerve (Stjärne 1966), and vas deferens (Austin *et al.* 1967). Some contradictory reports on the subcellular localization of this enzyme have appeared. Initially it was reported that tyrosine hydroxylase was largely particle bound (Nagatsu *et al.* 1964b, Nagatsu & Nagatsu 1970). The particle-bound enzyme appeared to be localized in nerve-ending fractions (synaptosomes) and probably in synaptic vesicles. On the other hand there are claims that tyrosine hydroxylase is exclusively localized in the soluble fractions of the adrenal medulla (Laduron & Belpaire 1968b,

Musacchio 1968) and splenic nerve (Stjärne & Lishajko 1967). Recent findings on the aggregation properties of tyrosine hydroxylase indicate that tyrosine hydroxylase is indeed a soluble enzyme. It appears that tyrosine hydroxylase forms aggregates and adsorbs particulate fractions under certain conditions during tissue homogenization (Musacchio *et al.* 1971, Wurzbürger & Musacchio 1971).

Inhibitors of tyrosine hydroxylase have been discovered which act by competing with the substrate, pteridine cofactor or Fe^{2+} . Hence such substrate analogues as α -methyl-p-tyrosine inhibit the enzyme in competition with tyrosine (Nagatsu *et al.* 1964b). Various catechol compounds including l-dopa, dopamine, noradrenaline and adrenaline inhibit the enzyme. The inhibition is competitive with the pteridine cofactor but uncompetitive with tyrosine (Udenfriend *et al.* 1965) and raises the possibility of a feedback inhibition of catecholamine biosynthesis. Tyrosine hydroxylase can also be inhibited by chelating agents such as α, α -dipyridyl (Nagatsu *et al.* 1964b) and ϕ -phenanthroline (Carlsson *et al.* 1963, Udenfriend *et al.* 1965). These compounds probably act by chelating Fe^{2+} . *In vivo* inhibitors of tyrosine hydroxylase lower the endogenous production of catecholamines in animals (Spector *et al.* 1965) and thereby bring about a reduction in the tissue levels of dopamine and noradrenaline.

Tyrosine hydroxylation is in fact established as the rate-limiting step in catecholamine metabolism by end-product noradrenaline inhibition (Nagatsu *et al.* 1964b, Levitt *et al.* 1965, Udenfriend 1966a). By competing with the pteridine cofactor it is thought that the free intraneuronal catecholamines control the rate of their own synthesis (Alousi & Weiner 1966, Ikeda *et al.* 1966), though a direct chemical oxidation of the pteridine cofactor to an inactive form cannot be excluded.

Dopa Decarboxylase (DDC) (EC 4.1.1.28)

This pyridoxal-requiring enzyme is not specific for l-dopa but is optically specific for aromatic l-amino acids such as 5-hydroxytryptophan, tryptophan, phenylalanine and tyrosine (Holtz *et al.* 1938, Lovenberg *et al.* 1962).

It is widely distributed, both intra- and extraneuronally; guinea pig and rat liver, guinea pig stomach, rat and dog brain and kidney tissue from a number of species all contain dopa decarboxylase (Lovenberg *et al.* 1962), as does the adrenal medulla (Langemann 1951), sympathetic nervous tissue (Goodall & Kirshner 1958, Holtz & Westermann 1956) and erythrocytes (Tate *et al.* 1971). It is present almost entirely in the cellular soluble fraction (Blaschko *et al.* 1955, Laduron & Belpaire 1968b), though Udenfriend (1966b) found that on careful homogenization of brain tissue as much as 60% of the dopa decarboxylase activity was found in the synaptosomes. It follows that if dopa decarboxylase is entirely localized within the cytoplasm then the enzymatically formed dopamine would have to be taken up by the storage vesicles which contain the dopamine- β -hydroxylase (Kirshner *et al.* 1963).

In an attempt to produce chemical sympathectomy at the dopa decarboxylase stage, a large number of inhibitors has been screened (Clark 1959, Sourkes 1966). This goal has, however, proved elusive due to the disseminated distribution of the enzyme throughout the body. The enzyme is present in such quantities that l-dopa does not accumulate, but is immediately converted to dopamine; little if any l-dopa can be detected in the blood or brain. Of the inhibitors studied, the one with the widest clinical application is l- α -methyldopa. This compound is a potent inhibitor of dopa decarboxylase *in vitro* (Sourkes 1954, Sourkes & d'Iorio 1963) and has catecholamine-depleting and hypotensive properties *in vivo* (Sjoerdsma *et al.* 1960). However it was subsequently found that these effects were not related to enzyme inhibition, but that l- α -methyldopa is itself decarboxylated by dopa carboxylase and it is the resulting amine that is

responsible for the release of tissue catecholamines and the observed hypotensive effect in man.

L-dopa is now wisely used in treating Parkinsonism (§8.2). Dopa decarboxylase inhibitors such as N-dl-seryl-N-(2,3,4-trihydroxybenzyl)-hydrazine (Ro4-4602), which inhibit the peripheral enzyme, but not the brain enzyme (Pletscher & Gey 1963), allow a 60-80% reduction in the required dose of l-dopa when the inhibitor and l-dopa are administered together (Rinne *et al.* 1972). The exact function of the extraneuronal dopa decarboxylase is not clear, though this could vary from one tissue to another. One explanation of why l-dopa is an effective agent in Parkinson's disease, even though extrapyramidal dopa decarboxylase appears to be lacking also, is that dopamine may be formed by the extraneuronal dopa decarboxylase near the receptor sites in the neostriatum of the brain. Other metabolic explanations have also been proffered (Wurtman *et al.* 1970).

Dopamine β -Hydroxylase (D β H) (EC 1.14.17.1)

Dopamine β -hydroxylase (which catalyzes the conversion of dopamine to noradrenaline) was first isolated and characterized in 1960 by Levin, Levenberg & Kaufman and subsequently purified to a single homogeneous protein (Friedman & Kaufman 1965b). Like tyrosine hydroxylase, it is a mixed function oxidase. Dopamine β -hydroxylase has broad substrate specificity with the structural characteristics necessary for β -hydroxylation being an aromatic ring with a side chain of two or three carbon atoms terminating in an amino group (van der Schoot & Creveling 1965).

The enzyme is ascorbate requiring (Levin *et al.* 1960) and contains copper (Friedman & Kaufman 1965a,b). It was found that the protein-bound copper was the site of the enzymatic reduction. Further study using electron paramagnetic resonance to probe the active site revealed that the Cu^{2+} was reduced by ascorbate; a large part of the reduced enzyme then being reoxidized during the substrate hydroxylation (Friedman & Kaufman 1966). These results support the concept of a reduction-oxidation role by the enzyme-

bound copper. However the exact role of the ascorbate co-factor has not been adequately explained. Liu *et al.* (1974) have presented evidence to suggest that the ascorbate may generate the superoxide anion (O_2^-) which in turn produces the active reduced enzyme.

Dopamine β -hydroxylase is an intraneuronal enzyme of the sympathetic nervous system and enzyme activity is found in the adrenal medulla (Levin *et al.* 1960), brain (Udenfriend & Creveling 1959) and in various sympathetically innervated organs. In the guinea pig the level of dopamine β -hydroxylase is very low at birth but reaches adult values within 5 days (Nagatsu *et al.* 1968). Unlike dopa decarboxylase this enzyme appears to be located exclusively in the storage vesicles of adrenergic nervous tissue (Kirshner 1957, Stjärne 1966, Stjärne & Lishajko 1967). About 50% of the dopamine β -hydroxylase in the catecholamine-containing granules of tissue homogenates is easily solubilized either by freeze-thawing or sonification. The rest of the enzyme activity is firmly attached to the granules (Kuzuya & Nagatsu 1969). During acetylcholine-stimulated secretion of catecholamines in perfused bovine adrenal glands dopamine β -hydroxylase is released into the perfusion medium together with the catecholamines (Viveros *et al.* 1968) (§2.5). It is thought that during secretion the entire contents of the storage vesicle are discharged directly to the exterior of the cell (Smith 1973).

Copper chelating agents such as diethyldithiocarbamate and alkylthiourea inhibit the enzyme *in vivo* and *in vitro*, reducing the level of tissue noradrenaline and increasing that of dopamine (Green 1964, Musacchio *et al.* 1964, Jonsson *et al.* 1967). Endogenous inhibitors which also act by chelating copper appear to be present in most tissues (Chubb *et al.* 1969). Substrate analogs (for example benzylhydrazine and benzyloxyamine) have proved to be potent inhibitors of dopamine β -hydroxylase (Creveling *et al.* 1962b, Nikodijevic *et al.* 1963). The well known drug reserpine inhibits this enzyme by blocking the dopamine-uptake mechanism of the amine storage vesicles (Kirshner 1962, Rutledge & Weiner 1967).

Phenylethanolamine N-methyltransferase (PNMT) (EC 2.1.1.28)

The final step in the conversion of tyrosine to adrenaline is the N-methylation of noradrenaline by phenylethanolamine N-methyltransferase. Bülbring (1949) demonstrated the methylation of noradrenaline in the adrenal gland and in initial studies with intact rats Keller *et al.* (1950) showed that the methyl group of ^{14}C -labelled methionine was incorporated into adrenaline synthesized in the adrenal medulla. The methyl group for the formation of adrenaline was subsequently shown to be from S-adenosylmethionine (Kirshner & Goodall 1957a).

In addition to noradrenaline, phenylethanolamine N-methyltransferase can methylate a variety of phenylethanolamine derivatives (Axelrod 1962). Phenylethylamines are, however, inactive with respect to the enzyme. It is also capable of methylating secondary amines and dimethylnoradrenaline has been found to be present in the adrenal medulla in small quantities (Axelrod 1960).

Phenylethanolamine N-methyltransferase is largely localized in the mammalian adrenal medulla, though small amounts have been located in the heart and brain (Axelrod 1962, Ciaranello *et al.* 1969, Pohorecky *et al.* 1969, Saavedra *et al.* 1974). The enzyme appears to be localized in the soluble fraction of the cell (Axelrod 1962), is absent from the early foetal adrenal gland and its appearance late in foetal life (Fuller & Hunt 1967) is accompanied by an increase in the adrenaline content of the gland (Roffi 1968).

The transferase is inhibited *in vitro* by its substrate noradrenaline and its product adrenaline. Since the inhibition by adrenaline occurs at concentrations normally present in the adrenal medulla this could be of physiological significance in the regulation of adrenaline synthesis (Fuller & Roush 1972). However since most of the adrenaline is sequestered in granules and presumably unable to interact with phenylethanolamine N-methyltransferase the degree of *in vivo* inhibition is unknown. Other

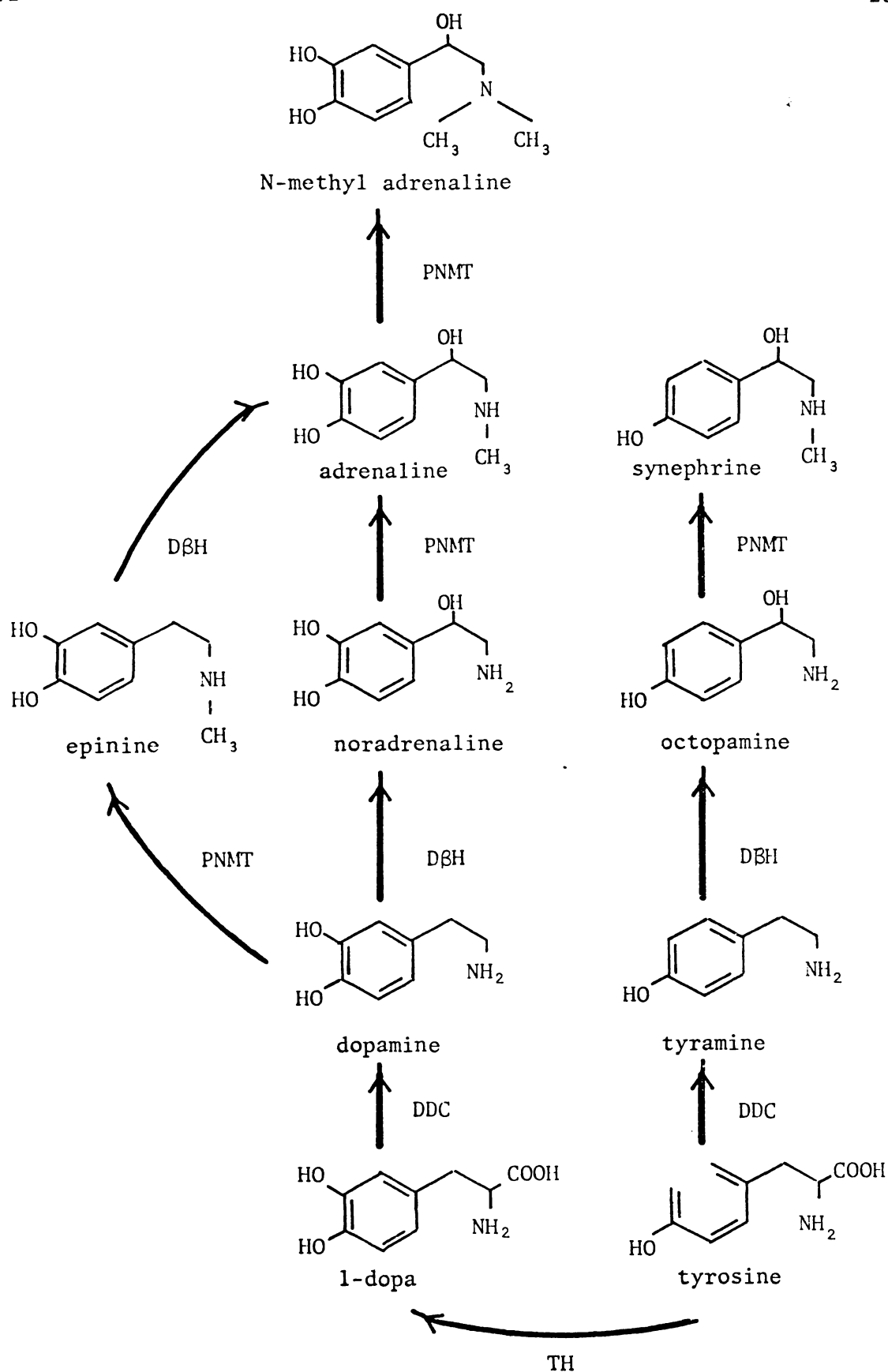


Figure 2-2. Possible alternative pathways in catecholamine biosynthesis.

inhibiting compounds include 2-cyclohexylcyclopropyl ammonium maleate and 8-amino-1,2-methanoindane-HCl (Krakoff & Axelrod 1967).

Although it is clear that the biosynthetic route proposed by Blaschko (1939) is the major pathway of catecholamine biosynthesis, lack of specificity of several of the enzymes permits several alternative pathways. One such pathway from tyrosine to noradrenaline and adrenaline may exist via tyrosine, tyramine, octamine and synephrine (Creveling *et al.* 1962a, Axelrod 1963) (Figure 2-2). Low levels of radioactively labelled noradrenaline and normetanephrine have in fact been detected in the urine of rats injected with ^{14}C -tyramine or ^{14}C -octamine (Creveling *et al.* 1962a). Furthermore octopamine and synephrine occur in human urine (Pisano *et al.* 1961, Kakimoto & Armstrong 1962). The physiological significance of this side pathway is not clear, but it may be significant in evaluating the pharmacological actions of tyramine (Creveling *et al.* 1962b).

2.3 REGULATION OF CATECHOLAMINE BIOSYNTHESIS

That the formation of catecholamines depends upon functional activity has been suspected for 70 years. Elliot (1905) noted that it was difficult to deplete the adrenal medulla by stimulation of the splanchnic nerve; an observation that suggested secretory activity might affect catecholamine formation.

An increase in the rate of release and synthesis of noradrenaline occurs as a result of increased adrenergic nerve activity. This occurs normally during exercise (von Euler *et al.* 1955, Gordon *et al.* 1966) and exposure to the cold (Oliverio & Stjärne 1965, Gordon *et al.* 1966, Rubenson 1969) or can be induced experimentally during electroconvulsive shock (Kety *et al.* 1967) or immobilization stress (Rubenson 1969). Even prolonged sympathetic nerve stimulation fails to deplete and in some cases even increases the endogenous noradrenaline and results in an increase in the urinary excretion of noradrenaline and its metabolites (Costa

et al. 1966). It is known that the turnover of adrenaline in the adrenal medulla is slow. Pendleton *et al.* (1973) have calculated a half life of 5.4 days. Thus only a small portion of adrenaline is released under basal conditions. On the other hand, Holland & Schümann (1956) and Bygdeman & von Euler (1958) reported that when the isolated adrenal gland is stimulated electrically or chemically it can secrete, within a few hours, amounts of adrenaline which are larger than can be accounted for by a simple depletion mechanism.

It is now recognized that enzymes in general are not passive biochemical tools: their activity is related to the demands of their environment. The manipulation of the catecholamine-synthesizing enzymes, both with respect to their activity and quantity, is the key to the control of catecholamine synthesis. However the availability of substrates and cofactors as well as the amount and availability of those enzymes could also be of importance. At present little is known about the factors that influence the rate of uptake of tyrosine from the bloodstream, the supply of cofactors, or the supply of enzymes to the terminals from the cell body.

As has already been mentioned (§2.2), noradrenaline is thought to participate in the regulation of its own synthesis by a feedback inhibition of tyrosine hydroxylase (Alousi & Weiner 1966, Ikeda *et al.* 1966), the rate determining step in the overall pathway (Nagatsu *et al.* 1964b, Levitt *et al.* 1965, Udenfriend 1966a). Dopamine may also inhibit this conversion (Udenfriend *et al.* 1965). Evidence in support of this hypothesis has come from Spector *et al.* (1967) who studied noradrenaline synthesis in brains and hearts of guinea pigs treated with an inhibitor of monoamine oxidase (a catecholamine-metabolizing enzyme). Noradrenaline accumulated in these tissues until it reached 2-3 times the concentration in untreated animals. In the treated animals the conversion of radioactive tyrosine to noradrenaline was reduced, but the formation of noradrenaline from labelled l-dopa was not affected. Working with the isolated hypogastric nerve-vas

deferens preparation of the guinea pig Weiner and his co-workers (Alousi & Weiner 1966, Weiner & Rabadjija 1968) showed that the increased rate of synthesis of ^3H -noradrenaline from ^3H -tyrosine associated with nerve stimulation could be blocked by adding noradrenaline to the perfusing fluid. With ^3H -l-dopa as the starting material no such effect was noted. Neither the total noradrenaline nor the amount of tyrosine hydroxylase activity was different in the stimulated preparation from the control.

It was proposed by these workers that nerve stimulation leads to reduction in the concentration of noradrenaline which can interact with tyrosine hydroxylase. This results in the activation of the normally inhibited enzyme and the consequent acceleration in noradrenaline synthesis. The postulated regulatory pool of noradrenaline would presumably be in equilibrium with intravesicular noradrenaline. This pool would need to be small enough for it to become depleted during and immediately after release of the vesicle contents. The concentrations of the free noradrenaline and of the pteridine cofactor present in the nerve terminal are not known but are probably less than 10^{-3}M , at which level they cause a 50% inhibition of tyrosine hydroxylase *in vitro* (Udenfriend *et al.* 1965). Recent work with added noradrenaline and pteridine cofactor (Weiner *et al.* 1972) indicates that the increased synthesis of noradrenaline from tyrosine associated with acute nerve stimulation is not simply the result of reduced end-product inhibition which is competitive with the pteridine cofactor. They suggested that it could result from reduced inhibition of the enzyme as a consequence of reduced levels of free intraneuronal noradrenaline, but that the effect could be more significant at an allosteric site on the enzyme or at some site where the interaction is not competitively antagonized by the pteridine cofactor.

A second possible site of endogenous inhibition of adrenaline synthesis is at the dopamine β -hydroxylase level. A sulphhydryl-containing compound resembling glutathione in amino acid composition has been found to inhibit dopamine β -hydroxylase *in vitro*

(Duch & Kirshner 1971). Finally there is the possibility of an adrenaline feedback inhibition of phenylethanolamine N-methyl transferase (Fuller & Roush 1972) (§2.2). In both cases no evidence exists to substantiate *in vivo* inhibition. In this connection however, Louis *et al.* (1974) noted that Parkinson's disease patients administered large quantities of l-dopa had a very high excretion of dopamine and its metabolites but not of noradrenaline. If the tyrosine hydroxylase step was the only regulatory step it might have been expected that the administration of l-dopa would lead to the formation of high tissue levels of noradrenaline and would increase the excretion of noradrenaline and its metabolites.

Long term control of catecholamine synthesis involves changes in enzyme quantities. A wide variety of pharmacological agents which, either directly or indirectly, bring about enhanced frequency of adrenergic nerve stimulation, can produce an increase in tyrosine hydroxylase and dopamine β -hydroxylase as well as phenylethanolamine N-methyl transferase in the adrenal medulla (Mueller *et al.* 1969a, Viveros *et al.* 1969) and in adrenergic tissues (Thoenen *et al.* 1969, Molinoff *et al.* 1970). Insulin hypoglycaemia is associated with an increase in tyrosine hydroxylase activity within 12 hours (Weiner & Mosimann 1970). The increase in tyrosine hydroxylase and dopamine β -hydroxylase can be prevented, at least partially, by inhibitors of protein synthesis (Mueller *et al.* 1969b, Kvetnařský *et al.* 1971b). The drug and insulin-induced elevation of these enzymes is probably a function of increased neural activity since the increase is prevented by decentralizing the ganglion or by the administration of ganglion blocking agents (Thoenen *et al.* 1969, Molinoff *et al.* 1970). Similarly the increase in tyrosine hydroxylase can be prevented by adrenal denervation (Thoenen *et al.* 1969). It is presumed that chronic nervous activity leads to the induction of the biosynthetic enzymes in some, as yet unknown, manner. A diminution of catecholamine-synthesizing enzymes as a possible consequence of decreased neuronal activity has also been observed. For instance mice raised in isolation show diminished tyrosine hydroxylase and

phenylethanolamine N-methyl transferase activity in the adrenals (Axelrod *et al.* 1970).

The recognition of a hormonal effect on the biosynthesis of adrenaline stems from observations by Shepherd & West (1951) and Coupland (1953), who noted that chromaffin cells not in contact with adrenal cortical tissue contained predominantly noradrenaline. This led to the suggestion that the formation of phenylethanolamine N-methyl transferase depends upon corticosteroid hormones. In fact hypophysectomy results in a marked decrease in the activity of phenylethanolamine N-methyl transferase (Wurtman & Axelrod 1966), a fall in the content of adrenaline in the adrenal medulla and the secretion of adrenaline following insulin-induced hypoglycaemia (Wurtman *et al.* 1967). The activity of phenylethanolamine N-methyl transferase can be restored by corticotrophin or by administering very large doses of glucocorticoids (Wurtman & Axelrod 1966). The amount of glucocorticoids required is far in excess of the normal replacement dose but may be consistent with the steroid concentrations normally delivered to the adrenal medulla from the cortex via the adrenal portal circulation (Wurtman 1966, Wurtman *et al.* 1972). When foetal rats are hypophysectomized by decapitation neither adrenaline nor phenylethanolamine N-methyl transferase is present in the newborn rat adrenal (Margolis *et al.* 1966). Tyrosine hydroxylase (Mueller *et al.* 1970) and dopamine β -hydroxylase (Weinshilboum & Axelrod 1970) also decline following hypophysectomy. The decrease in tyrosine hydroxylase activity is restored to normal by corticotrophin administration (Mueller *et al.* 1970) but not by amounts of glucocorticoids sufficient to completely restore phenylethanolamine N-methyl transferase activity, raising the possibility of a direct action by corticotrophin. While most of this research has been carried out with rats recent clinical observations support the existence of a similar mechanism in man (Duckworth *et al.* 1974). Inhibition of protein synthesis with puromycin or actinomycin D prevents the restoration of phenylethanolamine N-methyl transferase, which indicates that the steroids may act by inducing the formation of additional enzyme (Wurtman & Axelrod 1966).

Repeated daily immobilization stress in rats leads to an increase in the activity of tyrosine hydroxylase, dopamine β -hydroxylase and phenylethanolamine N-methyl transferase (Kvetnaňský *et al.* 1970, 1971a,b). If the animals are hypophysectomized before the first immobilization, the activity of all three enzymes is decreased. Immobilization then results in an increase in tyrosine hydroxylase activity but has no effect on phenylethanolamine N-methyl transferase activity (Kvetnaňský *et al.* 1970). The increased tyrosine hydroxylase and dopamine β -hydroxylase activity in both hypophysectomized and intact rats is abolished by adrenal denervation. Reis *et al.* (1970) observed an increase in phenylethanolamine N-methyl transferase in the adrenal glands of cats following attack behaviour evoked by hypothalamic stimulation; the increase was prevented by denervation.

The findings in general indicate that the catecholamine synthesizing enzymes are regulated by several factors which differ in importance from one enzyme to another. Thus hormonal influences probably predominate in the regulation of phenylethanolamine N-methyl transferase, whereas in the case of tyrosine hydroxylase, neuronal impulses are more important. Dopamine β -hydroxylase seems to be controlled by both factors to a similar extent.

2.4 CATECHOLAMINE METABOLISM AND UPTAKE MECHANISMS

The catecholamines are, in the main, inactivated by O-methylation and deamination by the enzymes catechol-O-methyltransferase and monoamine oxidase respectively. For many years it was believed that deamination by monoamine oxidase was the dominant route of catecholamine metabolism. However with the development of monoamine oxidase inhibitors it was soon realized that other pathways must be involved (Griesemer *et al.* 1953). In 1957 Armstrong *et al.* discovered large amounts of 3-methoxy-4-hydroxymandelic acid in the urine of patients with catecholamine-forming tumours (pheochromocytoma), suggesting that O-methylation might be a pathway for the metabolism of the catecholamines. The administration

of noradrenaline led to the excretion of large quantities of a 3-methoxy metabolite of noradrenaline, normetanephrine (Axelrod 1957), subsequently found to be a constituent of normal urine (Axelrod *et al.* 1958). At the same time, the O-methylating enzyme, catechol-O-methyltransferase was identified (Axelrod 1957, Axelrod & Tomchick 1958). In Figure 2-3 the principal pathways of catecholamine metabolism are outlined.

Catechol-O-Methyltransferase (COMT) (EC 2.1.1.6)

Catechol-O-methyltransferase is widely distributed in mammalian tissues, localized mainly in the soluble fraction of the cell (Axelrod & Tomchick 1958). In most animals the highest activity is found in the liver and kidney. It is present in all areas of the central nervous system, in varying concentrations, and in peripheral nerves (Axelrod *et al.* 1959a).

The enzyme requires Mg^{2+} but a divalent ion, including Co^{2+} , Mn^{2+} , Ni^{2+} , Fe^{2+} , Ca^{2+} and Zn^{2+} , may be substituted *in vitro*. S-adenosylmethionine is the methyl donor (Axelrod & Tomchick 1958). The highly purified enzyme has a molecular weight of about 24 000-29 000 (Anderson & d'Iorio 1968, Assicot & Bohuon 1970). Catechols are methylated but not monohydroxy derivatives of phenylethylamine. Naturally occurring compounds O-methylated by catechol-O-methyltransferase are dopa, dopamine, noradrenaline, adrenaline, 3,4-dihydroxymandelic acid, 3,4-dihydroxyphenylacetic acid, 3-hydroxyestradiol and ascorbic acid (Axelrod & Tomchick 1958, Knuppen *et al.* 1969). The electrophilic methyl group of S-adenosylmethionine is transferred to the hydroxy group by a nucleophilic displacement reaction. In the case of the catecholamines, where the meta-hydroxy group is strongly nucleophilic, the transfer takes place on the meta position. *In vivo*, O-methylation occurs almost exclusively on the meta position (Axelrod *et al.* 1958), though with the purified enzyme both meta- and para-substitution may occur, depending on the polarity of the substrate and the pH (Senoh *et al.* 1959). Catechol-O-methyltransferase is inhibited by sulphydryl binding reagents such as p-chloromercuribenzoate, indicating an active sulphydryl group. Polyphenyls

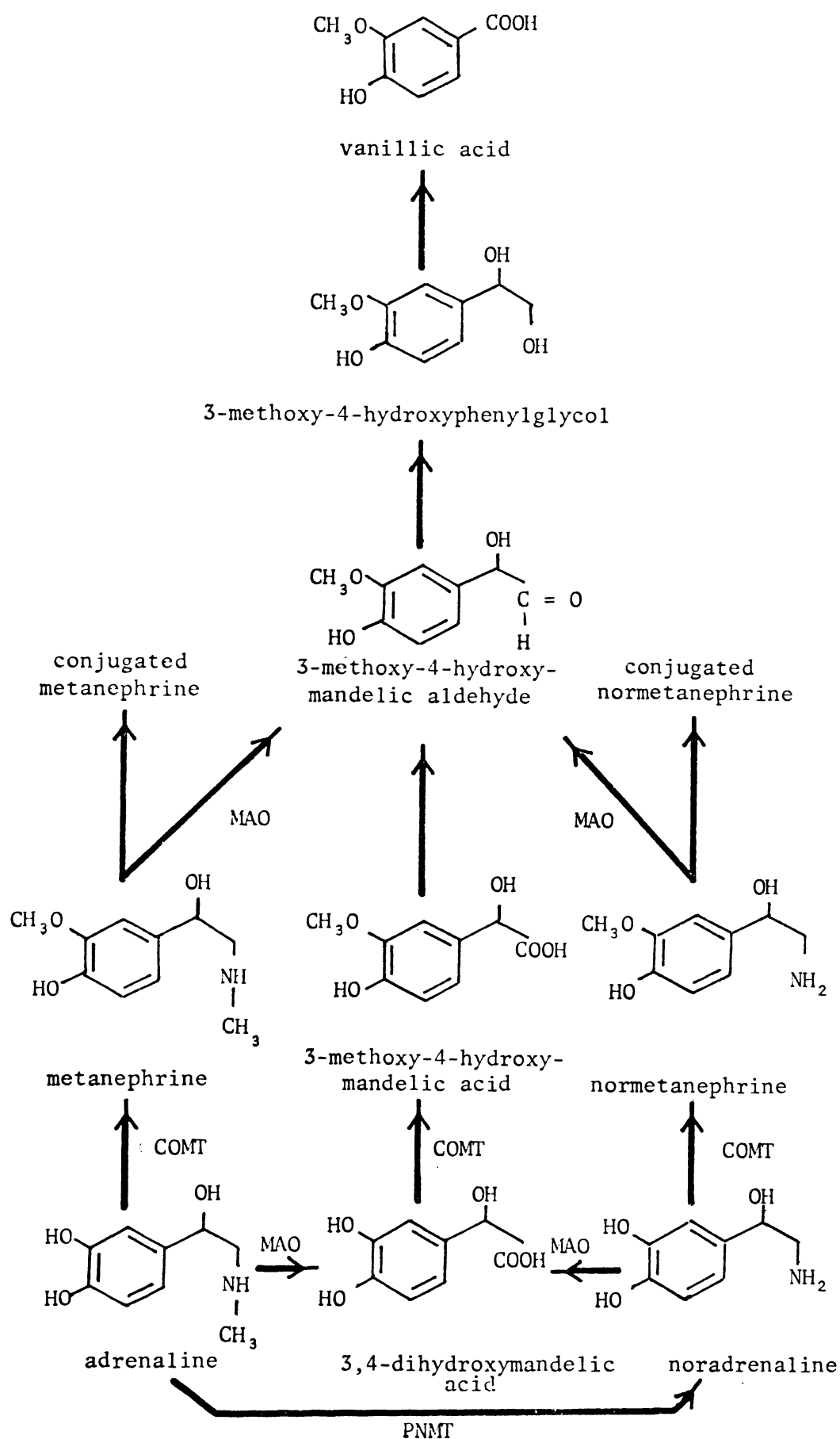


Figure 2-3. Principal catecholamine metabolic pathways.

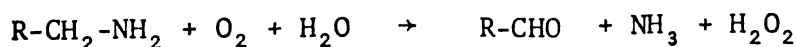
such as pyrogallol can inhibit the enzyme reversibly (Axelrod & LaRoche 1959). Flavoroids such as myricetin are also inhibitors, though in this case there are at least 3 structural groups on the molecule capable of inhibiting catechol-O-methyltransferase (Borchardt & Huber 1975).

Catechol-O-methyltransferase is present mainly outside the sympathetic neurone (Axelrod 1966), in contrast to monoamine oxidase which is largely an intraneuronal enzyme (Snyder *et al.* 1965). When the rabbit and cat vas deferens are denervated approximately 60% and 30% respectively of the catechol-O-methyltransferase disappears, indicating an intraneuronal location (Jarrott & Iversen 1971). The enzyme has also been found in close juxtaposition to the adrenergic receptors (Eisenfeld *et al.* 1967a, Eisenfeld *et al.* 1967b, Cuatrecasas *et al.* 1974). Until recently the extra-neuronal catechol-O-methyltransferase was considered to be at least partly extracellular, but there is now evidence to suggest otherwise. S-adenosylmethionine is exclusively intracellular, and blockers of noradrenaline uptake inhibit the formation of normetanephrine from either infused (Eisenfeld *et al.* 1967a,b) or nerve-released noradrenaline (Langer 1968).

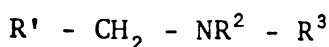
Monoamine Oxidase (MAO) (EC 1.4.3.4)

Monoamine oxidase was first described in 1928 by Hare who showed that tyramine could be deaminated *in vitro* to form p-hydroxybenzaldehyde, peroxide and ammonia. Monoamine oxidase can deaminate a wide spectrum of alkyl and aromatic amines including noradrenaline and adrenaline (Blaschko *et al.* 1937) and serotonin (Freyburger *et al.* 1952). Oxidative deamination by monoamine oxidase results in the formation of an aldehyde according to the equation

MAO



Aldehydes formed by the action of monoamine oxidase can be demonstrated *in vitro*, but are rarely detected in tissues or urine. They are immediately metabolized either to an acid metabolite by the enzyme aldehyde dehydrogenase (Leeper *et al.* 1958) or to an alcohol by the not yet clearly defined enzyme alcohol dehydrogenase. In the case of phenylethanolamine derivatives the action of this latter enzyme yields a 1,2-diol or glycol. Monoamine oxidase will oxidize primary, secondary and tertiary monoamines of the general formula.



where R^2 and R^3 may be hydrogen or methyl groups. The amino group must be attached to an unsubstituted CH_2 group. However N-methylation and β -hydroxylation decrease the susceptibility of phenylethanolamines to monoamine oxidase (Blaschko 1952).

Monoamine oxidase is a flavoprotein where flavin-adenine dinucleotide is the cofactor involved though the strength of attachment appears to be species dependent (Erwin & Hellerman 1967, Walker *et al.* 1971, Youdim & Sourkes 1972). The molecular weight of the enzyme appears to be about 10 000 but it may aggregate producing apparent weights of up to 100 000 (Tipton 1973).

There have been many suggestions that this enzyme exists in more than one form. Pharmacological evidence that multiple forms of monoamine oxidase exist (Gorkin 1966, Squires 1968) has been confirmed by the electrophoretic separation of several species of monoamine oxidase from rat liver and brain (Youdim *et al.* 1969, 1970). On the other hand, in some tissues such as the monkey small intestine (Murali & Radhakrishnan 1970) the enzyme appears to be homogeneous, exhibiting only a small band of activity on polyacrylamide-gel electrophoresis. Little is known about the nature of the phenomenon. Both conformational differences (Collins *et al.* 1970) and polymerization of active subunits (Gomes *et al.* 1969) have been suggested as a basis of explanation. Alternatively, the existence of multiple forms could be the result of attachment of membranous debris or varying amounts of

phospholipid (Verevkina *et al.* 1964). However since the enzymes appear to be localized in specific tissues, apparently have preferred substrates, and can be inhibited differentially with drugs it has been postulated that such multiple forms might have different physiological roles in the deamination of various biogenic amines (Sandler & Youdim 1972).

Many types of monoamine oxidase inhibitors have been used since the introduction of the hydrazine derivative iproniazid (Zeller & Barsky 1952). Other classes of monoamine oxidase inhibitors are amines containing the acetylene group, cyclopropylamines and β -methylphenylamines (Pletscher 1966, Pletscher *et al.* 1966). Administration of inhibitors results in an elevation of tissue levels of noradrenaline, octopamine (Molinoff *et al.* 1971), dopamine and serotonin (Spector 1963). The excretion of deaminated metabolites of catecholamines such as 3-methoxy-4-hydroxymandelic acid, 3-methoxy-4-hydroxyphenylglycol and 3-methoxy-4-hydroxyphenylacetic acid decreases while that of normetanephrine, octopamine and tyramine increases (Sjoerdsma 1966). Monoamine oxidase inhibitor drugs are currently used to treat hypertension and to alleviate depression. Although their mechanism of action is unclear, modified amine metabolism is presumed to be responsible for their activity.

Monoamine oxidase has been found in all vertebrates examined (Blaschko 1952). In man, the parotid and submaxillary glands have the highest activity (Strömblad 1959, Harada *et al.* 1971) but high concentrations are also found in the liver, kidney and in nervous tissues (Snyder *et al.* 1965). Blood platelets also contain the enzyme, or at least one form of it (Collins & Sandler 1971). Subcellular studies have indicated that the enzyme is highly localized in the outer mitochondrial membrane (Cotzias & Dole 1951, Schnaitman *et al.* 1967). In a number of innervated tissues the enzyme has been shown to be present both intraneuronally and extraneuronally. The loss of 50% of the monoamine oxidase activity that follows denervation of the vas deferens has been interpreted to indicate that about half of the enzyme is

located within the sympathetic nerves, with the remainder occurring in the extraneuronal cells (Jarrott & Iversen 1971); and similar results have been used to infer the presence of extraneuronal monoamine oxidase in the heart (Horita & Lowe 1972).

In connection with the evidence presented in §2.3 on the regulation of the levels of the biosynthetic amines it is interesting to note that monoamine oxidase activity is decreased by glucocorticoids and increased by adrenalectomy (Callingham & Lavery 1973, Parvez & Parvez 1973). Increased nervous activity in the vas deferens depresses the activity of the monoamine oxidase (Bhagat *et al.* 1973), presumably in response to the lowered CA concentration.

The overall roles of catecholamine-O-methyltransferase and monoamine oxidase in the inactivation of catecholamines are complex. After the release of noradrenaline from the sympathetic nerve most of the liberated catecholamine is inactivated by reuptake into the nerve terminal (Hertting & Axelrod 1961) (Figure 2-4). If labelled catecholamine is injected into an animal some of the labelled compound is taken up and retained unchanged in adrenergically innervated tissues (Axelrod *et al.* 1959b) where it remains long after the physiological effects have disappeared. In fact neither of the metabolizing enzymes is responsible for the rapid inactivation of noradrenaline. When both enzymes are inhibited, the action of noradrenaline is only slightly prolonged (Crout 1961a). Physical rather than metabolic termination may also be the mode of inactivation of serotonin and catecholamines in the central nervous system, but this is not easy to demonstrate. The ability of post-ganglionic neurons of the sympathetic nervous system to take up and retain catecholamines has been termed uptake₁ by Iversen (1975). Tissue catecholamine accumulation is markedly reduced if the sympathetic innervation is destroyed by surgical (Hertting *et al.* 1961) or immunologic means (Iversen *et al.* 1966) and parallels the decline in the tissue catecholamine content. A separate transport mechanism to uptake₁ exists for the transfer of free catecholamines from the axoplasm to the storage vesicle

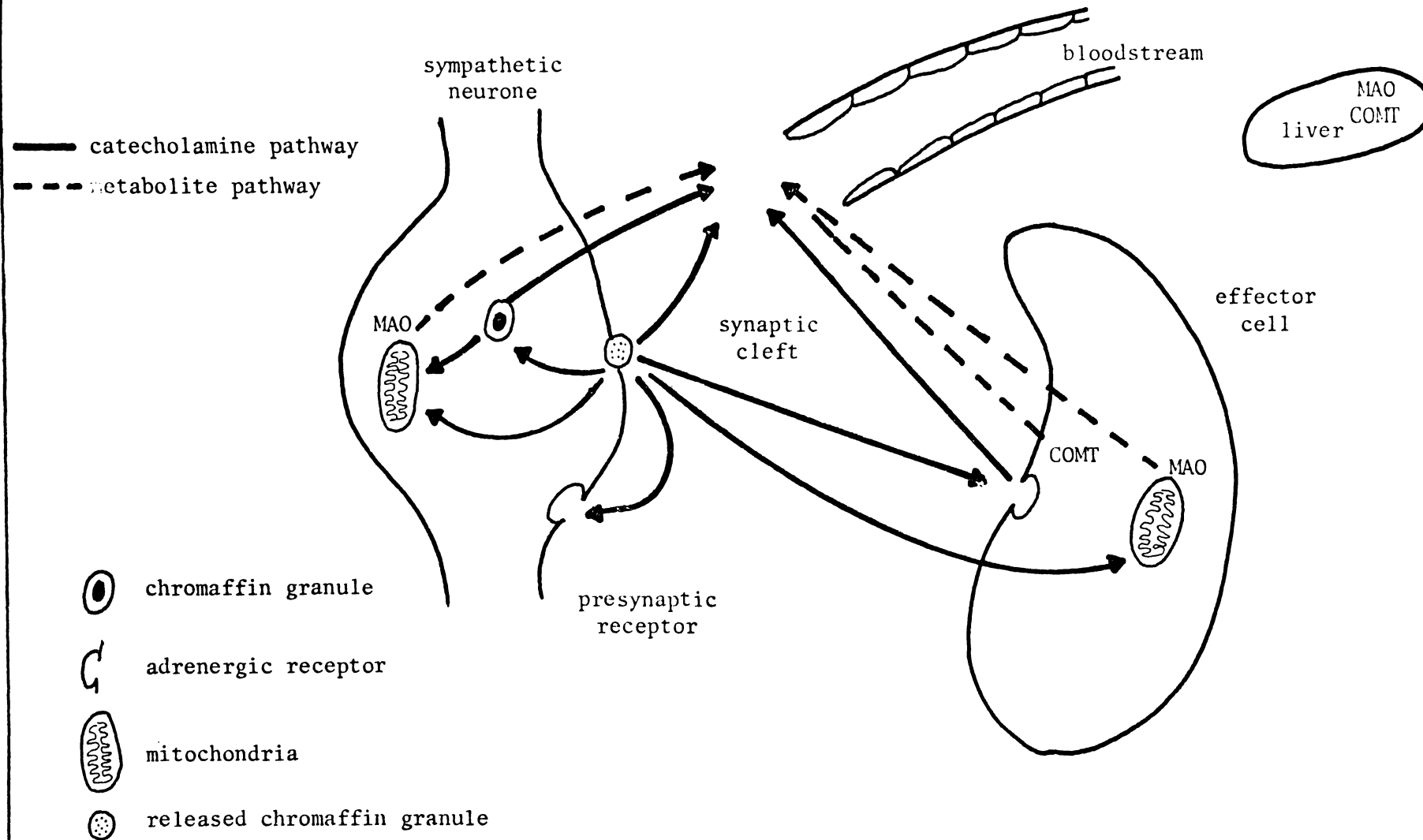


Figure 2-4. Catecholamine and metabolite pathways within the sympathetic neurone and after release into the synaptic cleft.

(Potter 1967) from where re-release may occur in response to subsequent sympathetic stimulation. A wide variety of amines that are structurally related to noradrenaline can be transported by the uptake system including both dopamine (Hellmann *et al.* 1971) and adrenaline (Iversen 1963), though the uptake process is less efficient than for noradrenaline. Compounds possessing the chemical properties necessary for uptake and granule storage but lacking in adrenergic receptor agonistic activity are termed false neurotransmitters (Kopin 1968).

In neural tissue the function of presynaptically localized monoamine oxidase may be to metabolize released neurotransmitter after reuptake into the nerve endings or after leakage from intraneuronal storage vesicles. Trendelenburg *et al.* (1972) have shown that the reuptake process itself depends on the presence of active monoamine oxidase in the nerve terminals. Under conditions in which monoamine oxidase is inhibited the capacity of the nerve endings to take up noradrenaline becomes exhausted, the storage vesicles become saturated and the level of free intraneuronal amine rises. Clarke & Sampath (1973) however, have found evidence indicating that monoamine oxidase deamination is dependent upon tyrosine hydroxylase activity, which supports the school of thought that monoamine oxidase activity preferentially deaminates newly synthesized amine rather than that removed from the synaptic cleft. The exact functional role of intraneuronal monoamine oxidase remains to be elucidated.

A small fraction of the released noradrenaline is O-methylated by catechol-O-methyltransferase outside the nerve and another small fraction is discharged into the bloodstream (Figure 2-4). The binding of ^3H -noradrenaline to receptor cells at the synaptic cleft appears to be to a membrane-bound catecholamine-binding protein which is closely related to catechol-O-methyltransferase (Cuatrecasas *et al.* 1974). There is considerable evidence to indicate the presence of a presynaptic α -adrenergic receptor which inhibits depolarization-induced release of noradrenaline from the post-ganglionic sympathetic neurone (Langer 1974, Berthelsen & Pettinger 1977). Once released into circulation (and this

includes all adrenal medulla catecholamine discharge) the amines are susceptible to inactivation by catechol-O-methyltransferase and/or monoamine oxidase, particularly in the liver and kidney. One probable function of the extraneuronal monoamine oxidase is the destruction of potentially toxic amines which are ingested, and the particularly high levels of activity in the liver, stomach and intestine may be important in this respect (Blaschko 1952).

The uptake of catecholamines by peripheral extraneuronal sites (uptake₂) has a higher affinity for adrenaline than noradrenaline. Uptake₂ is associated with rapid metabolic degradation by monoamine oxidase and catechol-O-methyltransferase (Iversen 1975). The ability of vascular smooth muscle to take up and metabolize catecholamines may be of particular importance to the fate of circulating catecholamines in view of the sparse sympathetic innervation of the tissue (Iversen 1975). There is also good evidence to suggest that extraneuronal uptake and metabolism of catecholamines in a variety of other tissues is important in restricting the access of circulating catecholamines to the receptors of effector organs (Trendelenburg 1978). Where dense adrenergic innervation is present extraneuronal uptake has little influence on the fate of noradrenaline released by adrenergic nerve endings. An extraneuronal uptake mechanism has been reported for noradrenaline in rat brain (Hendley *et al.* 1970), and presumably such noradrenaline would be deaminated in monoamine oxidase-containing cells. In general however, the quantities of deaminated, but not O-methylated, noradrenaline and adrenaline metabolites (3,4-dihydroxyphenyl-glycol and 3,4-dihydroxymandelic acid) excreted in the urine are small (Kopin & Axelrod 1960, Weil-Malherbe 1967).

Maas & Landis (1971) have analysed the kinetics of the metabolism of infused noradrenaline in order to obtain solutions for a model representing the fate of noradrenaline released from the sympathetic nerves into the circulation in man. They concluded that free noradrenaline, conjugated noradrenaline and normetanephrine excreted into the urine are derived from circulating noradrenaline

and those metabolic pools in equilibrium with circulating noradrenaline. Most of the urinary 3-methoxy-4-hydroxymandelic acid appears to originate in pools which, relative to their size or rate of noradrenaline synthesis, are poorly penetrated by circulating noradrenaline. These pools might represent the brain, the adrenal medulla and/or the peripheral nerve noradrenaline. As a result of this and other radioactive studies *in vivo* and *in vitro* it has been concluded that, in general, the overflow of noradrenaline released from sympathetic nerve endings is metabolized mainly by O-methylation, and that noradrenaline metabolized within the nerve endings is predominantly deaminated by monoamine oxidase (Kopin & Gordon 1962, 1963).

The methoxylated amine derivatives of the catecholamines are all substrates for monoamine oxidase and can thus give rise to acid or alcohol metabolites bearing the 3-methoxy-4-hydroxyphenyl group. Catechol-O-methyltransferase can also methylate acid or alcohol metabolites of the catecholamines. Thus both monoamine oxidase and catechol-O-methyltransferase are fully active on the products of the other, giving rise to an entire spectrum of metabolites (Appendix I), some acted upon by monoamine oxidase, others by catechol-O-methyltransferase and still others by both.

Phenols are generally detoxicated in animals by conjugation of the phenolic hydroxyl group with sulphuric or glucuronic acid, and indeed it is found that catecholamines are largely excreted into the urine as the sulphate or glucuronide. While sulphate conjugates are predominant in man (Richter 1940, Elmadjian *et al.* 1956, Sharman 1973) glucuronide conjugation is predominant in the rabbit and rat (Dodgson & Williams 1949, Clark *et al.* 1951, Ceasar *et al.* 1969).

The relative amounts of the main catecholamine metabolites that appear in human urine after the intravenous administration of radioactive dopa, dopamine, noradrenaline and adrenaline appear in Table 2-1. However the interpretation of these results is subject to the appreciation that the metabolism of exogenously

Table 2-1

The Percentage Distribution of the Metabolic Products
found in Human Urine after Intravenous Administration
of L-dopa, Dopamine, Noradrenaline and Adrenaline

	Substance Infused			
	L-dopa	Dopamine	Noradrenaline	Adrenaline
L-dopa	1.4	-	-	-
3-Methoxy-tyrosine	3.6	-	-	-
Dopamine	16.5	3.4	-	-
Conjugated	6.4	1.5	-	-
3-Methoxy-tyramine	2.4	Trace	-	-
Conjugated	1.6	6.3	-	-
Noradrenaline	Trace	1.5	4-16	-
Conjugated	-	2.4	Up to 8	-
Normetanephrene	Trace	0.2	3	-
Conjugated	-	4.5	(Sulphate 13 (Glucuronide 5	-
Adrenaline	-	Trace	-	2- 7
Metanephrene	-	-	-	3-12
Conjugated	-	-	-	(Sulphate 30-39 (Glucuronide 1-6
MHPG	-	(MHPG + MHPE) conjugates	(Free + con- jugated) 13 sulphate 10	(MHPG + DHPG) 2
Conjugated	-	4.2		Sulphate 3-6
DHPG	-	-	-	-
Conjugated	-	-	Sulphate 8	-
MHPE	Conjugate 1.3	) 0.3	-	-
DHPE	-		-	-
VMA	0.7	7.7	32	25-41
Conjugated	1.2	-	-	-
DOMA	Trace	0.6	2	0.6-2
Conjugated	-	-	-	0.9
HVA	18.0	27.5	-	0.8
Conjugated	1.7	-	-	-
DOPAC	8.6	2.0	-	1.7
Conjugated	2.1	-	-	-
Vanillic acid	-	-	4	1-2

The figures represent percentages of the radioactivity found in the urine of human beings up to 96 hours after an intravenous injection or infusion of a radioactive catecholamine. [from Sharman 1973]

administered catecholamines is different from that of endogenously released catecholamines. Further, catecholamine metabolism is species dependent (Merits *et al.* 1973) though the range of catecholamine metabolites is common to many species - even the chicken excretes a similar range of catecholamine metabolites to many mammals (Rennick *et al.* 1965). 3-methoxy-4-hydroxymandelic acid accounts for over 90% of the normal catecholamine and catecholamine metabolite excretion in the human adult. The mean value of the 24 hour urinary catecholamine excretion are: for men noradrenaline - 24.3 ± 6.9 (SD) μg , adrenaline - 7.1 ± 2.9 μg , 3-methoxy-4-hydroxymandelic acid 6.9 ± 2.1 mg; for women noradrenaline - 23.1 ± 7.1 μg , adrenaline - 5.6 ± 2.4 μg , 3-methoxy-4-hydroxymandelic acid 6.2 ± 2.5 mg (Becker & Kreuzer 1970). Significant quantities of dopamine and its metabolites also appear in the urine (Ceasar *et al.* 1969, Karoum *et al.* 1969). The principal metabolite of dopamine is 3-methoxy-4-hydroxyphenylacetic acid or homovanillic acid, a product of monoamine oxidase and catechol-O-methyltransferase and aldehyde dehydrogenase. Some of these metabolites come from the brain but most probably arise from dopamine which has been acted on by monoamine oxidase before it could be protected by uptake into the storage vesicles.

The catabolism of catecholamines in the central nervous system has been studied by determining the substances formed in the brain after the administration of radioactively labelled catecholamines or their precursors, by examining the metabolites formed from catecholamines in brain slices *in vitro* and by estimating some of the metabolic products formed from the endogenous catecholamines *in vivo*. In the rat Glowinski *et al.* (1965) found that ^3H -noradrenaline given via the lateral ventricle, was metabolized to normetanephrine, was deaminated and also formed O-methylated, deaminated metabolites. Studies with slices of rabbit brain *in vitro* (Rutledge & Jonason 1967) showed that radioactive dopamine was mainly converted to 3,4-dihydroxyphenylacetic acid and homovanillic acid, together with a small amount of 3-methoxytyramine. Noradrenaline was metabolized in this system to the two glycols 3-methoxy-4-hydroxyphenylglycol and 3,4-dihydroxyphenylglycol. Endogenous 3,4-dihydroxyphenylacetic acid

(Rosengren 1960) and homovanillic acid (Andén *et al.* 1963, Sharman 1963) have also been demonstrated in brain tissue.

2.5 THE RELEASE OF THE CATECHOLAMINES

In the normal mammalian adrenal, chromaffin cells are innervated by cholinergic, preganglionic, sympathetic nerves which traverse the thoracic and upper lumbar parts of the sympathetic chains. It is clear that these nerve fibres play by far the dominant role in regulating the secretory activity of the chromaffin cells of the adrenal medulla. Acetylcholine was shown to be the normal physiological mediator of excitation of the adrenal medulla (Feldberg & Minz 1933) and both nicotinic and muscarinic receptors are present in the gland (Feldberg *et al.* 1934). Following denervation chromaffin cells discharge only small amounts of catecholamines (Vogt 1952), though the discharge is increased under conditions of anoxia or in response to drugs having a direct systemic effect on chromaffin cells. Numerous secretagogues and physiological conditions are known to increase catecholamine discharge from sympathetic nerves and the adrenal medulla. In Table 2-2 the more important are listed with representative references.

There are many similarities between the mechanisms of release of catecholamines from the adrenal medulla and from peripheral nerve terminals, but it is not yet certain whether the two processes are identical. Three events may be considered to comprise the cycle of events leading to catecholamine release - excitation, release of the product, and recovery. Douglas & Rubin (1961a) introduced the expression *stimulus-secretion coupling* to encompass the chain of events initiated by the action of acetylcholine on the adrenal medullary cells and which results in the secretion of adrenaline and noradrenaline. In common with the secretory processes of many glands Ca^{2+} is essential to stimulus-secretion coupling (Rubin 1970). Calcium deficient perfusion solutions depress or abolish the secretory response of the adrenal medulla to splanchnic nerve stimulation, acetylcholine and a variety of other secretagogues (Douglas & Poisner 1962, Rubin *et al.* 1967,

Table 2-2

Chemical Agents, Environmental and Experimental Conditions and Disease States Associated with a High Level of Catecholamine Release

Acetylcholine	Feldberg <i>et al.</i> (1934) Douglas & Rubin (1963)
Angiotensin II	Peach & Ober (1974)
Histamine	de Schaepdryver (1959)
Nicotine	Douglas & Rubin (1961b)
Caffeine	Poisner (1973)
Some anaesthetics	Emmelin & Strömlad (1951) Price (1959)
Insulin hypoglycaemia	Weiner <i>et al.</i> (1960)
Work/exercise	Vendsalu (1960) Banister & Griffiths (1972)
Low temperatures	Wilkerson <i>et al.</i> (1974)
High temperatures	Alvarez & Johnson (1973)
Hypoxia/high altitude	Richtarik <i>et al.</i> (1966) Kotchen <i>et al.</i> (1973)
Pain/surgery	von Euler (1954) Walker <i>et al.</i> (1959) Coward & Smith (1966)
Mental concentration and stress	von Euler & Lundberg (1954) Elmadjian <i>et al.</i> (1958) Taggart <i>et al.</i> (1973)
Emotional arousal	Levi (1965)
Low blood pressure and/or volume	Zileli <i>et al.</i> (1974)
Splanchnic stimulation	Bülbring & Burn (1949) Silver (1960)
CNS stimulation	de Schaepdryver (1959)
Immobilization	Bliss <i>et al.</i> (1968) Welch & Welch (1968)
Some mental disorders	Schildkraut & Kety (1967)
Phaeochromocytoma	Geffen <i>et al.</i> (1973a)
Hypertension	Geffen <i>et al.</i> (1973b)
Cardiac infarction	McDonald <i>et al.</i> (1969a)
Hypocapnia/acidaemia	Tenney (1956) Millar & Morris (1961)

Borowitz 1969). Increasing the Ca^{2+} concentration in the perfusion fluid increases the duration of catecholamine release from the adrenal medulla (Borowitz 1971). Excess potassium increases Ca^{2+} uptake, probably as a result of a depolarizing action, and this leads to an increased catecholamine release (Douglas & Rubin 1961a, Baker & Rink 1975). Drugs such as caffeine, d-amphetamine or chlorpromazine which can increase secretion even in the absence of Ca^{2+} may exert their effect by mobilizing intracellular calcium (Rahwan *et al.* 1973b). It was initially proposed that the depolarization of the cell plasma membrane associated with acetylcholine release permits the entry of calcium into the cell initiating the secretory process (Douglas & Rubin 1961a, Douglas & Poisner 1962, Douglas & Rubin 1963). However it was subsequently demonstrated that depolarization and Ca^{2+} entry into the cell are not tightly coupled events and it is now thought that the depolarization is more likely to be a consequence of the increased membrane permeability (Douglas *et al.* 1967a,b). In the sympathetic neurones too it appears that the link between depolarization and release is the influx of Ca^{2+} into the nerve terminal (Smith & Winkler 1972). After this shift in calcium distribution however our knowledge of the sequence of events is largely based on speculation.

Five basic hypotheses have been proposed for adrenal medullary secretion.

1. Interaction of acetylcholine with the cell membrane in the presence of Ca^{2+} increases the membrane permeability to the 'free' cytoplasmic catecholamines which diffuse out of the cell. The concentration of the 'free' pool is maintained by diffusion from the storage vesicles.
2. Stimulation causes a change in the permeability of the storage vesicle membrane resulting in an increase of the concentration of 'free' catecholamines which then diffuse out of the cell.

3. Stimulation results in an extrusion of the intact storage vesicles which then disrupt outside the cell liberating their contents.
4. Stimulation initiates a sequence of events resulting in release of the storage vesicle contents directly to the exterior of the cell with the retention of the vesicle membrane within the cell (*exocytosis* or *reverse pinocytosis*).
5. The vesicles or the contents of the vesicles are emptied into a complex system of microtubules and thence expelled to the cell exterior.

The first two of these mechanisms may be ruled out on the grounds that the efflux of noradrenaline from the cytosol is not increased by nerve stimulation (Häggendal & Malmfors 1969, Farnebo & Hamberger 1971), the spontaneous efflux of noradrenaline from the cytosol is much slower than that evoked by nerve stimulation and the temperature-dependence of diffusion and the observed release are different (Lindmar & Loeffelholz 1972). Furthermore it might be reasonably expected that in an animal not treated with a monoamine oxidase inhibitor, most of the noradrenaline or adrenaline passing from the vesicle into the cytosol would be oxidized by the monoamine oxidase of the mitochondria, leaving the neurone as deaminated metabolites (§2.4).

Hence mechanisms which place the vesicle as the principal source of the released amine must be considered. The vesicles contain catecholamines, ATP, and a number of proteins called *chromogranins* (Hillarp *et al.* 1955, Hillarp 1958, Blaschko & Helle 1963, Banks & Helle 1965, Blaschko *et al.* 1967). Since dopamine β -hydroxylase is stored in these vesicles (§2.2) it is, by definition, a chromogranin. Indeed the amino acid sequence of chromogranin A (the major protein of the vesicle soluble species) and dopamine β -hydroxylase appear identical, though the secondary structure may differ since the two proteins are immunologically distinct (Aunis *et al.* 1975). Mechanisms of the third and fourth types

have received powerful support from the observations of Douglas and co-workers (Douglas *et al.* 1965, Douglas & Poisner 1966a,b) that upon stimulation of perfused cat adrenal glands, adenine nucleotides and their breakdown products were released simultaneously with catecholamines. Furthermore the molar ratio of catecholamine to adenine nucleotides in the perfusates was close to that existing in the intact storage vesicles (4:1). Subsequent studies by others have demonstrated that the chromogranins, including chromogranin A (Banks & Helle 1965, Kirshner *et al.* 1967) and dopamine β -hydroxylase (Viveros *et al.* 1968, Smith *et al.* 1970), are also concomitantly released with the catecholamines upon nervous stimulation and in the same relative amounts that are present in the water-soluble fraction of the granule. Cytoplasmic enzymes such as tyrosine hydroxylase are not detectable in the perfusates. Blaschko *et al.* (1967) collected adrenal venous blood from anaesthetized calves during and between periods of stimulation of the splanchnic nerve and found that the ratio of catecholamine to chromogranin A during periods of stimulation was similar to that reported by Schneider *et al.* (1967) for the isolated perfused gland. As in the perfused gland the appearance of the protein lagged behind that of the catecholamines. This lag has been attributed to the slower rate of diffusion of the protein through the interstitial spaces and vasculature.

As well as these biochemical data, evidence indicative of exocytosis has been shown by electron microscopy of thin sections (de Robertis & Sabatini 1960, Coupland 1965b). Morphological evidence is however difficult to obtain, probably owing to the infrequency with which the fusing vesicle and plasma membrane coincide with the plane of a thin section. More recent work with freeze-fractured adrenal medullary chromaffin cells (Smith *et al.* 1973) has provided more definite visual proof of exocytosis. The sites of exocytosis were abundant on stimulated cells but rare when calcium was omitted from the system. Acetylcholine caused a 3-fold increase in the number of vesicles fused to the cell membrane (Smith & Van Orden 1973).

While the exocytosis hypothesis enjoys considerable experimental support it does not satisfactorily account for the fast rate of discharge of secretory products that is observed (Schramm 1967). This has prompted the proposal that there exists within secretory cells a complex system of contractile secretory tubules to facilitate the egress of secretory products. Evidence in support of this latter hypothesis is the existence of an actinomyosin-like protein in the adrenal medulla (Poisner 1970), and the fact that catecholamine secretion is inhibited by labilizers of microtubules and microfilaments and increased by microtubule stabilizers (Poisner & Bernstein 1971). Such microtubules have recently been visualized by electron microscopy (Rahwan *et al.* 1973a). The similarities in molecular mechanisms of secretion and muscle contraction, particularly with regard to the calcium requirement, lend further support to the possibility that secretion may take place within contractile secretory tubules rather than by exocytosis.

Whatever the exact mechanism of catecholamine release, dopamine β -hydroxylase is released by nerve stimulation and with the development of a sensitive plasma dopamine β -hydroxylase assay (Molinoff *et al.* 1970) it has been suggested that the activity of the enzyme in plasma may be an index of sympathetic nervous activity (Weinshilboum & Axelrod 1971, Algate & Leach 1978). In rats subjected to repeated stress by immobilization there was an increase in plasma dopamine β -hydroxylase (Weinshilboum *et al.* 1971). This increase was found even after adrenal gland demedullation, indicating that increased enzyme activity present after stress is mainly of sympathetic nerve origin. However this view has been challenged by Reid & Kopin (1974) who found little correlation between plasma noradrenaline and dopamine β -hydroxylase activity under various drug regimes. Dixon *et al.* (1975) also failed to establish any link between catecholamine release from the stimulated perfused cat adrenal and dopamine β -hydroxylase activity in the perfusate.

2.6 ADRENERGIC RECEPTORS

The fact that adrenergic stimuli could produce both excitatory and inhibitory responses in different tissues puzzled physiologists for many years following the turn of this century.

Ahlquist (1948) concluded that there must be two types of adrenergic receptor, but they could not be classified simply as inhibitory or excitatory. Instead he arbitrarily designated two receptor types (α and β) based on the two main orders of potency of 6 adrenergic agonists. The updated definition of an α receptor according to Furchgott (1972) is one *which mediates a response pharmacologically characterized by*

- (1) *a relative potency series in which adrenaline > or = noradrenaline > phenylephrine > isoprenaline*
- (2) *a susceptibility to specific blockade by phentolamine, dibenamine or phenoxybenzamine at relatively low concentrations*

while the β receptor is one which mediates a response pharmacologically characterized by

- (1) *a relative potency series in which isoprenaline > adrenaline > noradrenaline > phenylephrine (or isoprenaline > noradrenaline > adrenaline > phenylephrine)*
- (2) *a susceptibility to specific blockade by either propranolol or pronethalol at relatively low concentrations*

Evidence subsequent to Ahlquist's proposal has shown that the β receptor population is not homogeneous (Lands *et al.* 1967) and can be differentiated into two groups termed β_1 and β_2 receptors. Lands *et al.* (1967) compared the effectiveness of 15 sympathomimetic amines in causing bronchodilation (guinea-pig perfused lung), vasodepression (anaesthetized dog), cardiac stimulation (rabbit heart) and lipolysis (rat adipose tissue). The relative potencies of the various compounds suggested that the cardiac and lipolytic responses were controlled by a receptor (β_1) which differed from the type (β_2) involved in bronchodilation and vasodepression. Some doubts have however been expressed about the interpretation

of these and other data and the experimental procedures involved (Furchgott 1972, Jenkinson 1973). The adrenergic receptor classification as it presently is conceived should therefore be used with caution. Subdivision of α receptors into two classes has also been advocated (Berthelsen & Pettinger 1977, U'Prichard & Snyder 1979). Adrenaline is a potent activator of α , β_1 and β_2 receptors while noradrenaline is a more selective activator of α and β_1 receptors than it is of those of type β_2 (Moran 1975).

It should be emphasized that the differentiation of receptors in the manner described is an operational procedure based on the pharmacological characterization of responses mediated by receptors. This does not necessarily mean that the receptors differ in chemical composition. It is possible for example that the response of an adrenergic receptor is modified by the surrounding molecules in the macromolecular structure, such as the cell membrane with which they may be associated (Ahlquist 1973). The β adrenergic receptor has proved difficult to identify and isolate because of the comparatively low affinity exhibited towards the catecholamines (Levitzki *et al.* 1974) and the presence of non-receptor catecholamine-binding proteins including a system functionally related to catechol-O-methyltransferase (Cuatrecasas *et al.* 1974). The precise location of adrenergic receptors in the cell is also unknown, although the rapidity of several responses to catecholamines would suggest the receptors are on the outer surface of the cell membrane (Lefkowitz *et al.* 1973). Dopamine has complex effects - as well as activating specific dopamine receptors it may also weakly activate both α and β receptors (Goldberg 1972).

The catecholamine-adrenergic receptor interactions are only the first step in the mediation of cellular responses to the catecholamines. In many cells increased β adrenergic stimulation is associated with augmented activity of adenyl cyclase and increased production of cyclic adenosine 3',5'-monophosphate (Sutherland & Robison 1966, Lefkowitz *et al.* 1976) though probably not all actions of the catecholamines are mediated through this effector mechanism.

THE CATECHOLAMINES: METHODS OF ASSAY

CHAPTER 3

3.1 INTRODUCTION

With the widespread interest in the catecholamines by biochemists, physiologists, pharmacologists, psychologists and endocrinologists the development of appropriate assay procedures has absorbed much effort over the last 25 years. The large number of publications related to the assay of catecholamines indicates that the search for methods of sufficient precision and sensitivity is continuing. In this review the general methods of isolating and measuring 1-dopa, dopamine, noradrenaline and adrenaline and, where appropriate, some of their metabolites will be considered and some attempt made to evaluate the sometimes-conflicting claims of the various authors.

Tissue levels of catecholamines, particularly in the central nervous system (§1.3) may give valuable information on the activity of catecholamine-containing structures and methods have been developed for their extraction and determination in a number of tissues. However, as we have already seen (§2.3), there are powerful mechanisms in operation tending to oppose changes in the concentration of catecholamines in neural tissue and it is usually preferable to measure turnover rates rather than absolute levels (Persson & Waldeck 1970). Free and conjugated catecholamines are also found in the urine and reflect to some extent the state of the sympathetic nervous system. Urinary catecholamines together with certain metabolites are not difficult to determine because of their presence in comparatively high concentrations though interference by other urinary substances can pose problems.

Such measurements have proved to be an informative and reliable procedure in many catecholamine studies. Nevertheless, even with frequent collections, urinary levels represent an integration of plasma catecholamine levels over time. Depending on the type of study envisaged urinary catecholamine levels representing damped plasma levels can be a handicap since catecholamine secretion is subject to numerous secretagogues and psychological stimuli (§2.5). The interpretation of plasma catecholamine levels is also subject to many considerations in view of the lability of these compounds both in time and in space (§5.1).

Generally the steps in the assay of catecholamines may be divided into

- (1) sample collection and extraction
- (2) purification and separation of the different catecholamines
- (3) estimation.

3.2 SAMPLE COLLECTION AND EXTRACTION

The methods developed for the purposes of extracting, concentrating and purifying the catecholamines from biological samples are based usually on solvent-extraction, ion-exchange, aluminium oxide adsorption or on various combinations of these procedures. Solvent-extraction is generally restricted to the analysis of tissue amines, while ion-exchange or aluminium oxide adsorption are the preferred methods for the analysis of body fluids. Since the catecholamines are susceptible to biological and atmospheric oxidation (especially in the presence of traces of heavy metal catalysts) or enzymatic degradation, precautions are necessary in sample collection and preparation. Hökfelt (1951) reported that while the catecholamine content of the adrenal glands and the liver of the sacrificed rat remained constant for up to 24 hours at 20°, the concentration in the heart declined by 50%. In the brain the noradrenaline content markedly declines following death. For the mouse brain the decrease at 5, 15, 30 and 60 minutes and at 4 hours after decapitation (at 25°) was 10,

21, 30, 44 and 69% respectively. Instantaneous, irreversible inactivation of catecholamine catabolizing enzymes by sacrificing mice using microwave irradiation gives brain levels of catecholamines close to those found in mice killed by decapitation, with sample removal and freezing within several minutes (Weintraub *et al.* 1975). In this respect tissue catecholamines are less labile than other neurotransmitters including acetylcholine (Stavinoha & Weintraub 1974). In the rat brain the decreases at 30 and 60 minutes and at 4 hours were 15, 28 and 53% respectively (Grabarits *et al.* 1966).

Extracts of tissues suitable for adsorption or further extraction procedures may be obtained by homogenizing a weighed sample in cold perchloric or trichloroacetic acid (Bertler *et al.* 1958). When cation exchange resins are to be used in purification of perchloric acid extracts the excess perchloric acid may be removed as the relatively insoluble potassium salt. It is not feasible to extract catecholamines in their un-ionized form into organic solvents owing to their sensitivity to oxidation in alkaline media (Fernando 1972). Extraction in a cationic form requires a sufficiently polar solvent such as acidified n-butanol (Weil-Malherbe & Bone 1954, Montagu 1956, Ansell & Beeson 1968; Maickel *et al.* 1968, Haubrich & Denzer 1973, Schlumpf *et al.* 1974). Other solvent systems reported in the literature include butanol-saturated sodium chloride solution (10:1) (Shore & Olin 1958), acidified ethanol (Crawford & Outschoorn 1951), and acidified acetone (Fleming *et al.* 1965). Heptane is frequently added to the solvent extract to facilitate transfer to the aqueous phase (Fleming *et al.* 1965, Shore & Olin 1958, Maickel *et al.* 1968, Haubrich & Denzer 1973, Metcalf 1974, Schlumpf *et al.* 1974). It should be noted that extraction with solvents is somewhat unspecific and to avoid high blanks further purification is usually necessary. Many authors employ direct aluminium oxide adsorption (§3.3) of the acid extract.

For the assay of catecholamines in urine the recommended procedure is to collect the daily urinary output in a vessel containing 15 ml of 6N hydrochloric acid (Crout 1961b) or hydrochloric acid and ascorbic acid (Fernando 1972). The final pH should be adjusted to about 3-4 as the content of free catecholamine in this range shows insignificant changes in a refrigerator (+4°) or freezer (-20°) over a period of more than 3 months (Andersson *et al.* 1974). Considerable increases in the concentration of free nor-adrenaline are observed at pH values below 1 due to amine release from hydrolyzed conjugates (von Euler & Lishajko 1961b, Andersson *et al.* 1974). At a higher pH (4-8) and 4° the catecholamine concentration decreases owing to microbial decomposition.

To determine total urinary catecholamines the usual approach is to hydrolyze the conjugates by heating at 100° for 20-30 minutes at a pH between 0 and 1.5 (von Euler & Orwén 1955, Weil-Malherbe & Bone 1957b). This procedure appears to be satisfactory for the hydrolysis of the sulphates but it does not readily split the more stable glucuronides. Nevertheless only a small proportion of catecholamines and their metabolites in man are glucuronides and may be neglected in human studies (LaBrosse & Mann 1960, Sharman 1973). A further objection to acid hydrolysis lies in its destructive effect which increases with the time of heating and with increasing acidity. In the case of some acid metabolites such as 3-methoxy-4-hydroxymandelic acid and 3-methoxy-4-hydroxyphenylglycol this problem is particularly serious. Finally, acid hydrolysis gives rise to high blanks and further purification becomes necessary.

The alternative, enzymatic hydrolysis with β -glucuronidase and arylsulphatase (Takahashi & Gjessing 1972) is a milder procedure, but also entails some problems which have generally restricted its use to the analysis of certain catecholamine metabolites (Weil-Malherbe 1971).

1. The available preparations are inhibited by sulphates and phosphates present in urine.
2. The prolonged incubation at 37° necessary for quantitative hydrolysis is a cause of the poor recovery often obtained.
3. Some enzyme preparations contain mucins not precipitated by trichloroacetic acid or perchloric acid, which can clog adsorption columns or cause the formation of emulsions during extraction procedures. Furthermore it has been pointed out that commercial preparations of these enzymes are heavily contaminated by dopamine and l-dopa (Anton *et al.* 1973).

While it is clear that the major mechanisms for the removal of catecholamines from circulation lie outside the blood, catecholamine activity in whole blood decreases on standing. Manger *et al.* (1959) reported a decline of 11% in 10 minutes and Carruthers *et al.* (1970) a 40% decline in 30 minutes. The enzymes monoamine oxidase (§2.4), catechol-O-methyltransferase (§2.4) and dopamine β -hydroxylase (§2.2) are all present in blood. Catechol-O-methyltransferase is localized in erythrocytes both as a soluble (Axelrod & Cohn 1971) and a membrane-bound (Assicot & Bohuon 1971) form which differ in substrate affinities, pH optima and thermostabilities. In contrast to the soluble form the activity of the membrane-bound catechol-O-methyltransferase decreases as the immature erythrocytes (reticulocytes) develop. The membrane-bound enzyme appears to be functionally associated with an adrenergic β -receptor (Quiring *et al.* 1975). Catechol-O-methyltransferase is also present in leucocytes in trace amounts (Baldessarini & Bell 1966). Monoamine oxidase is present in blood platelets possibly comprised of several isoenzymes or containing multiple binding sites (Collins & Sandler 1971, Edwards & Chang 1975). Platelets are known to have an active uptake mechanism for catecholamines (Abrams & Solomon 1969, Born & Smith 1970). Recent studies have also revealed that considerable monoamine oxidase activity is present in reticulocyte mitochondria

(Quiring & Hubertus 1975). The level of the platelet enzyme is subject to a number of influences including age (Robinson 1975), sex (Roth *et al.* 1976), iron uptake (Youdim *et al.* 1975) drugs (Bockar *et al.* 1974) and endocrine status (Belmaker *et al.* 1974). In addition the enzyme activity is genetically determined (Wyatt *et al.* 1973). Human plasma also contains a soluble monoamine oxidase that deaminates a variety of monoamines including tyramine, tryptamine, dopamine and ethanolamine (McEwen 1965). This soluble form resembles intraneuronal monoamine oxidase in several respects but is inactive towards serotonin, noradrenaline and adrenaline while some evidence suggests that it is associated with connective fibre synthesis (McEwen & Castell 1967). In addition plasma contains dopamine β -hydroxylase (§2.2), released together with catecholamines from sympathetic nerve terminals and from the adrenal medulla (§2.5). The level of dopamine β -hydroxylase in rat (Kuzuya *et al.* 1976) and human (Freedman *et al.* 1972) plasma and in guinea pig adrenals (Nagatsu *et al.* 1968) is age dependent, is elevated under conditions of sympathetic arousal and may be either high (Geffen *et al.* 1973b) or low (Nishizawa *et al.* 1974) in different diseases.

In whole blood *in vitro* there is significant movement of noradrenaline and adrenaline, added in high concentrations, into erythrocytes (Roston 1966, 1967). With inhibition of catechol-O-methyltransferase by topolone Branco *et al.* (1974) were able to demonstrate that approximately 15% of added noradrenaline (final concentration $1 \mu\text{g ml}^{-1}$) was within blood cells after 20 minutes incubation. Roston (1967), using lower concentrations of radioactively labelled noradrenaline and adrenaline, found an even more rapid disappearance from blood plasma with 25% loss in 90 seconds, approaching 40% after 3 minutes. Over 30 minutes no further uptake was recorded. ^{14}C -noradrenaline removal from whole blood at 37.5° was characterized by a rapid fall in activity over 30 minutes followed by a slower phase with approximately 40% of the initial tracer within the blood cells at 1 hour (Zimon *et al.* 1966). Thus the uptake and the metabolism of catecholamines by enzymes within the formed elements of the blood appears to be an important pathway in the post-sampling loss of

catecholamine activity. The relative significance, if any, of the soluble plasma enzymes to assay results is unknown.

Little consideration appears to have been given to the effects of protein-binding of catecholamines on the interpretation of assay results, on differences between assays using protein-stripped plasma and those that do not, and on the implications of possible biological activity of bound catecholamines. Binding of circulating noradrenaline and adrenaline is known to occur: a low capacity, high affinity binding site has been identified and estimated to account for up to 50% of physiological levels of circulating noradrenaline (Danon & Sapira 1972). While 3,4-dihydroxymandelic acid and dopamine are competitors (albeit weaker than noradrenaline) for noradrenaline-binding sites on plasma proteins, substances with a 3-O-methyl group are not bound (May *et al.* 1974). Two different types of binding sites were reported by Russell & Doty (1973). A low affinity, temperature dependent binding site at approximately one hundred times the concentration of the high affinity site was also detected. With a lower activation energy of unbinding, catecholamines bound to this site are more readily available physiologically compared to the high affinity binding site. Binding reached almost 50% at very low concentrations of added adrenaline dropping to about 20% at 5 ng ml⁻¹ (incubation for 24 hours at 3° in charcoal-stripped plasma). Only 1-3% binding occurred in unstripped plasma. Conflicting results using whole dog blood have also been published (Branco *et al.* 1974). With the addition of noradrenaline to the blood at a final concentration of 50 ng ml⁻¹ only 54.5±20.6% (n=7) of the expected concentration was found by fluorimetry. Neither incubation time (2 to 20 minutes) nor temperature (20° and 37.5°) significantly altered the outcome. On the other hand bioassay gave results ranging from 81-97% of expected concentrations. At this noradrenaline concentration 41.3% of the noradrenaline added was found in the protein precipitate. Almost complete (95-100%) fixation of protein-bound catecholamine to the denatured protein has appeared to occur during acid precipitation (Russell & Doty 1973). On this point there is disagreement in the literature as

no loss of added ^3H -noradrenaline was found by Weise & Kopin (1976) while Diamant & Byers (1975) put their loss at $18 \pm 5\%$ after protein precipitation. Differences in centrifugation efficiency and incubation times may account for part of this discrepancy but further work is clearly required.

Since cellular uptake is markedly inhibited by cold (Abrams & Solomon 1969) refrigeration of whole blood samples reduces losses to a minimum. The addition of anti-oxidants such as sodium sulphite or ethylenediamine-tetraacetic acid (which complexes heavy metal catalysts and also acts as an anti-coagulant) prevents oxidation losses at this and subsequent stages. For reproducible results it is also necessary to achieve rapid plasma separation from the cellular fractions of blood. The plasma may then be frozen and stored for later analysis. While some authors maintain that negligible losses of catecholamines in plasma stored at -20° occur over 3 days (Jiang *et al.* 1973) losses of 50% and more were reported by Carruthers *et al.* (1970) over 11 days. It would generally appear advisable if immediate analysis is not possible, to precipitate plasma proteins with perchloric or trichloroacetic acid and extract (§3.3) before freezing. This reduces the pH of the samples to a level at which the catecholamines are more stable. Noradrenaline has a maximum stability at pH 4.0. Such extracts at -20° are stable for long periods of time (Griffiths *et al.* 1970, Andersson *et al.* 1974).

3.3 CATECHOLAMINE PURIFICATION AND SEPARATION

Some assays, including the enzymatic isotopic procedures of Passon & Peuler (1973) and da Prada & Zürcher (1976), the bioassay of Gaddum *et al.* (1949) and Fernando (1969) and the paper electrophoretic method of Eichhorn & Rutenberg (1965), avoid the need for extraction and purification by direct application to untreated plasma or urine and even to whole blood in the bioassay of Vane (1964). Nevertheless prior purification and, in many cases, prior separation of constituent catecholamines is essential to most procedures. The discovery that freshly prepared aluminium

oxide or hydroxide adsorbs catechol and catechol compounds in alkaline solution (Shaw 1938) was the crucial discovery in the development of methods for the extraction and purification of catecholamines from biological fluids. In a study of the factors influencing catecholamine adsorption onto aluminium oxide, Anton & Sayre (1962) developed the procedure currently used by most analysts. Not only the brand of aluminium oxide, but also its preparation procedure were found to markedly influence the final recovery. The adsorption did not occur below pH 7 and became maximal at pH 8.0-8.5 (Figure 3-1). High quality water and the use of sodium sulphite to prevent oxidation of the catecholamines in the extraction may also increase recoveries. Weil-Malherbe & Bone (1952) found that they obtained lower blanks, better recoveries, and faster rates of flow if they treated the commercial aluminium oxide with hot 2N hydrochloric acid, washed free of acid by repeated decantation and finally dried at 300° the optimal temperature for the preparation of highly active aluminium oxide (Russell & Cochran 1950). Drell (1970) re-examined the adsorption of catecholamines and their metabolites onto aluminium oxide and found that catecholamines and catecholamine acids are adsorbed at pH 4 only in low ionic strength solutions and at pH 6 only in the absence of phosphate ions. At pH 8.5 quantitative adsorption was confirmed even in solutions of high ionic strength or in the presence of phosphate ions.

Catecholamine adsorption onto aluminium oxide can be accomplished by two techniques.

1. Batch Technique (Griffiths *et al.* 1970)

A quantity of aluminium oxide (about 400 mg) and the specimen are mixed in a beaker and the pH adjusted to 8.5 with sodium hydroxide. Disodium ethylenediamine tetraacetic acid (200 mg) and sodium sulphite (10 mg) are added for protection of catecholamines in alkaline media. It is usually recommended that stirring be done with a stirring rod as opposed to using a magnetic stirring bar - to avoid grinding the aluminium oxide to a fine powder. The aluminium oxide, now carrying the adsorbed catecholamines is

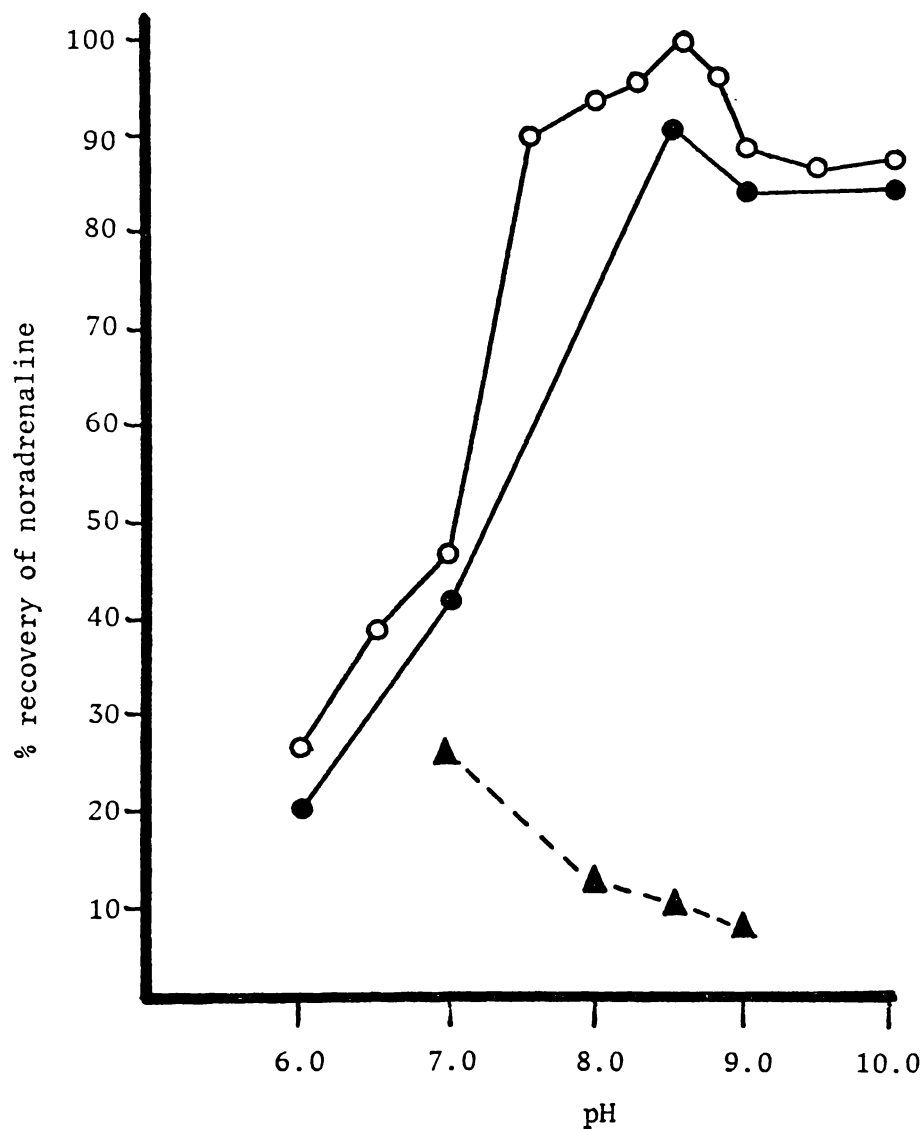


Figure 3-1. Effect of pH, metabisulphite and ethylenediamine tetraacetic acid (EDTA) on the recovery of noradrenaline (0.5 μ g in 25 ml 0.4 N perchloric acid) from aluminium oxide. [from Anton & Sayre 1962]

- acid made with EDTA-distilled water, with metabisulphite
- acid made with EDTA-distilled water, no metabisulphite
- ▲ acid made with non-EDTA-distilled water, no metabisulphite

washed several times in distilled water, the wash fluids decanted and the catecholamines eluted from the alumina by acid. Acetic acid (0.2-0.5 N), oxalic acid (0.6 N), hydrochloric acid (0.01-0.1 N), sulphuric acid (0.05-0.4 N) and perchloric acid (0.035-0.25 N) have been used. Since hydrochloric and sulphuric acids are claimed to dissolve small quantities of aluminium (whose precipitation in alkaline media may interfere with fluorimetric assays) acetic acid and perchloric acid are more widely used. In the assay of plasma catecholamines some concentration of the plasma extract is possible at this stage. Eluent volumes as low as 1.2 ml (O'Hanlon *et al.* 1970) and 1.0 ml (Vaage 1974) have been used.

2. Column Technique (Valori *et al.* 1969)

Here the aluminium oxide is adjusted to about pH 8.5 before or after placing in a small column. The specimen is brought to the same pH and the catecholamines are adsorbed as the solution passes over the aluminium oxide. The column can then be washed and eluted with acid. This procedure has theoretical and some practical advantages over the batch method. The adsorbed material should separate quantitatively in sharp bands on the column allowing removal of impurities and elution in a small volume. Moreover, washing and eluting can be carried out more efficiently, in less time, and with better recoveries on a column than by repeated batch washings involving centrifuging and decanting. In practice some authors (von Euler & Orwén 1955, Anton & Sayre 1972, Wood & Mainwaring-Burton 1975) maintain that column adsorption gives erratic results owing to difficulty in sustaining a constant flow pattern. The catecholamines are also exposed to an alkaline pH for a longer period of time.

Some authors, to combine the advantages of the two techniques, use batch adsorption and transfer the aluminium oxide with the adsorbed catecholamines to a column for elution (Crout 1961b, Fiorica 1965, Valori *et al.* 1969). Vaage (1974) used a filter tube to batch-adsorb the catecholamines from urine, removing the effluent and water wash through the sintered glass filter by

centrifuging. Column elution was accomplished in the same manner with 1 ml of 0.035 N perchloric acid. Typical recoveries using the batch technique are $78.0 \pm 5.0\%$ for noradrenaline and $79.1 \pm 5.0\%$ for adrenaline (Griffiths *et al.* 1970) while Anton & Sayre (1962) and O'Hanlon *et al.* (1970) achieved over 90% recovery. Using a column for elution $94.5 \pm 1.4\%$ of the noradrenaline and $93.5 \pm 1.9\%$ of the adrenaline was recovered by Valori *et al.* (1969).

Aluminium oxide purification of catecholamines is not specific: all catechol compounds are adsorbed. Thus l-dopa, dopamine, noradrenaline, adrenaline and all their catechol metabolites are retained to varying degrees. Some separation of the catecholamines from their acidic metabolites is possible using elution acids of varying strengths (Drell 1970).

Ion Exchange Resins

Cation-exchange resins have been used for the extraction and separation of catecholamines from tissue extracts or body fluids either instead of aluminium oxide, or for further purification - a step sometimes found necessary in the case of urine. Some analysts (Bertler *et al.* 1958, Häggendal 1962) prefer strongly acidic resins of the sulphonic acid type, but most prefer weakly acidic resins of the carboxylic type (Kirshner & Goodall 1957b, Martin & Harrison 1968, Viktora *et al.* 1968, Renzini *et al.* 1970, Seki & Wada 1975). Carboxylic resins have the advantage of greater ease of elution but are less efficient in amine retention and more sensitive to electrolyte concentration. It is advisable to reduce the concentration of electrolytes to a minimum in view of their competitive effects. A few millilitres of 1N acetic acid is all that is required for elution. Sulphonic resins require mineral acid at a strength of 1-2 N. These high concentrations can cause destruction of catecholamines and leach fluorescent contaminants from the resin itself (Häggendal 1962).

Apart from catecholamine purification, ion-exchange resins are used for separating dopamine, noradrenaline and adrenaline from each other and from their various metabolites. For many assay

methods, with limited resolution between individual catecholamines, good separation is essential. By lengthening the column a complete separation of the catecholamines and their derivatives can be achieved (Häggendal 1962), though at the expense of recovery and elution time. Both types of resin have been used to separate catecholamines from each other (Kirshner & Goodall 1957b, Bertler *et al.* 1958, Seki & Wada 1975) or from their metabolites (Häggendal 1962, Mattok & Wilson 1965, Lange *et al.* 1970).

Mattok & Wilson (1965) made use of the fact that boric acid forms complexes with catechols by eluting a weak cation-exchange column with 4% boric acid and then eluting the methylated derivatives with 2N sulphuric acid. Complete separation of urinary dopamine, nordrenaline and adrenaline on an Amberlite (Rohm & Haas Company, Philadelphia) IRC-50 resin is also possible using an eluent of acetate buffer (pH 5.6, 0.2 M) containing boric acid (0.67 M) and ethylenediamine tetraacetic acid (5 mM) mixed with *t*-butanol, tetrahydrofuran and 2,2'-thiodiethanol in proportions of 800:160:40:1 by volume (Seki & Wada 1975).

A combination of aluminium oxide and ion-exchange resins has also been used to separate the principal catecholamines and their metabolites. Masuoka *et al.* (1963) described the separation of 1-dopa, dopamine, noradrenaline, adrenaline, normetanephine, metanephine and 3-methoxytyramine by the use of aluminium oxide, Amberlite CG-50 and Dowex 50X8 (Dow Chemical Company, Midland, Michigan, USA) resins. Separation of urinary 3-methoxy-4-hydroxyphenylacetic acid (homovanillic acid), 3-methoxy-4-hydroxymandelic acid (vanilmandelic acid) 3,4-dihydroxyphenylacetic acid and 3,4-dihydroxymandelic acid was achieved by Messiha *et al.* (1973) using Dowex-Z-X4 for initial purification and basic aluminium oxide. Homovanillic acid was extracted from the Dowex-Z-X4 effluent with ethyl acetate. Homovanillic acid may also be separated from vanilmandelic acid using the anion exchange resin Dowex-1-X2 after initial separation from interfering substances and precursors on Dowex-50W (Shibuya *et al.* 1975). In the initial purification noradrenaline and dopamine are eluted separately using 0.8 N and 2N hydrochloric acid respectively.

Dalmaz & Peyrin (1976) separated the urinary 3-methoxy derivatives of dopamine, noradrenaline and adrenaline from each other in borate buffer on an Amberlite CG-50 column after purification on a Dowex-50W-X2 column while the acidic O-sulphate conjugates of 3-methoxy-4-hydroxyphenylglycol and 3,4-dihydroxyphenylglycol are separable on an intermediate base, anion exchange column (Stone & Mendlinger 1974).

Gel Filtration

Sephadex (Pharmacia Fine Chemicals AB, Uppsala, Sweden) G-25 is capable of resolving added catecholamines from plasma protein (Marshall 1963) while Sephadex G-10 with 0.02N acetic acid eluent was shown to completely separate l-dopa from dopamine, noradrenaline from adrenaline and normetanephrine from metanephrine (McDonald & Murphy 1973). Recovery was better than 90% but the samples were diluted 5-fold and elution time was 6 hours.

Thin Layer and Paper Chromatography

Thin layer and paper chromatography have been successfully applied to the separation of catecholamines and their metabolites and have proved to be especially useful in monitoring the metabolic fate of exogenously-administered, labelled catecholamines (Fleming & Clark 1970, Baumann *et al.* 1971, Breebaart *et al.* 1972, Harrison *et al.* 1973, Merits *et al.* 1973, Vohra & Jayasundar 1976). These procedures are not usually employed in analytical work owing to the time involved, the low concentrations usually present and to the difficulties in quantifying the separated compounds. Nevertheless Breebaart *et al.* (1972) have applied two-dimensional thin-layer chromatography to the analysis of catecholamine and serotonin-producing tumours, while Tsuzuki *et al.* (1972) employed a three-phase thin layer plate to first convert dopamine, noradrenaline and adrenaline to their dansyl derivatives and then to carry out a separation. With a scanning fluorimeter, the sensitivity lay between 0.02-2.0 µg. An alternative is to acetylate with acetic anhydride to yield the O,O-N-triacetyl derivatives which can be extracted with dichloromethane,

concentrated, and separated by paper chromatography (Sharman 1971). Takahashi & Gjessing (1972) employed thin layer chromatography to separate and purify urinary catecholamines prior to fluorescence quantitation.

Thin layer chromatography is essential to many radioenzymatic assays for the separation of the O-methylated products of catechol-O-methyltransferase (§3.10).

Some representative examples of the application of thin layer (TLC) and paper chromatography and the systems employed are presented in Table 3-1. High speed liquid chromatographic determination of catecholamines is covered in §3.8 and gas-liquid chromatography (GLC) in §3.9.

3.4 BIOLOGICAL ASSAYS

In the last 20 years chemical methods of assaying catecholamines have superseded and largely resulted in the abandonment of biological assay techniques. Although bioassay methods lack accuracy and specificity, and on the whole are difficult to set up and maintain, their sensitivity permits very small quantities to be assayed.

As a consequence of the diverse physiological effects of noradrenaline and adrenaline a number of approaches to the problem of measurement have been made. These include -

Blood Pressure

The pressure response of the pithed cat in response to the administration of the unknown, introduced by Elliot (1912) and extended to the rat (Shipley & Tilden 1947, Holzbauer & Vogt 1956, Muscholl & Vogt 1958, Fernando 1969), has been widely employed. The method is only suitable for the estimation of total catecholamines unless the components are first separated. In the pithed cat some discrimination for noradrenaline over adrenaline is shown;

Table 3-1

Thin Layer and Paper Chromatographic Systems for the Separation
of Catecholamines, their Precursors and Metabolites

Reference	Stationary Phase & Development Time	Solvent System (v/v)	Substances Separated	Detection System
Vahidi & Sankar (1969)	Whatman No.1 filter paper (3-24 hr)	butanol/HAc/H ₂ O (5:1:3) ethyl acetate/HAc/H ₂ O (5:1.5:3)	L-dopa DA NA A MN Tyramine HVA VMA	20% Folin phenol reagent in water and exposure to ammonia
	cellulose (2-5 hr)	ethyl acetate/HAc/H ₂ O/ butanol (3:1:3:2)		
Engelman & Portnoy (1970)	silica gel (2 hr)	butanol/formic acid/H ₂ O (15:2.5:1.5)	NMN MN	ultra-violet (UV) light
Fleming & Clark (1970)	microcrystalline cellulose (80 μ m thick) (3½-4 hr)	butanol/methanol/1N formic acid (60:20:20) (solvent system I for first dimension)	DOMA DHPG DOPAC HVA NMN MN MHPG MT	diazotized p-nitroaniline/
	(1½ -2 hr)	chloroform/methanol/1N NH ₄ OH (60:35:5) (solvent system II for second dimension)		

Table 3-1 (continued)

Reference	Stationary Phase & Development Time	Solvent System (v/v)	Substances Separated	Detection System
			Octopamine Tyramine TYR VMA L-dopa DA NA A	
Forrest <i>et al.</i> (1970)	Whatman No. paper discs (radial development)	(1) phenol/0.1N HCl (85:15) (2) butanol saturated with 2N HCl	A epinine adrenaline- methyl ether	(1) UV before and after ammonia (2) 4-aminoantipyrine and $K_3Fe(CN)_6$ (3) $K_3Fe(CN)_6$ and ammonia (4) ninhydrin
Baumann <i>et al.</i> (1971)	cellulose (2-2½ hr)	butan-2-one/acetone/2.5N HAc (40:20:20)	Tyrosine L-dopa DA NA NMN VMA	p-nitroaniline

Table 3-1 (continued)

Reference	Stationary Phase & Development Time	Solvent System (v/v)	Substances Separated	Detection System
Takahashi & Gjessing (1972)	cellulose (3¼-3½ hr)	butanol/HAc/H ₂ O (4:1:1)	DA NA A	ethylenediamine/ K ₃ Fe(CN) ₆ /ethanol UV
Harrison <i>et al.</i> (1973)	polyamide (45 minutes) (30 minutes)	chloroform/ethyl acetate/HAc (7:2:1) benzene/butan-2-one/methanol (6:2:2)	L-dopa and metabolites	fluorescein and UV
Cryer <i>et al.</i> (1974)	silica gel (3½ hr)	isopropanol/butanol/H ₂ O/ formic acid (60:20:19:1)	NMN MN	UV
de Prada & Zürcher (1976)	silica gel (25 minutes)	chloroform/ethanol/ethylamine (79%) (16:3:2)	MT NMN MN	UV
Vohra & Javasundar (1976)	cellulose (3-4 hr)	butanol/ethanol/0.5N HAc (35:8.5:20)	NMN DOMA DHPG MHPG VMA NA	water/ethylenediamine (1:4) UV

noradrenaline is about 3-4 times more active. With the pithed cat sensitivity ($0.1 \mu\text{g ml}^{-1}$) is adequate for urinary samples but not for plasma. Sensitivity may be increased by the injection of cocaine (Lewis & Waton 1957). With the pithed cat quantities as low as 0.5-1.0 ng may be estimated. Even lower quantities were sufficient to elicit a response from the pithed, deviscerated preparations of Venkatakrishna-Bhatt (1975).

Intact anaesthetized cats and rats have also been used (Lewis & Waton 1957, but sensitivity is low. Sensitivity of a much higher order has been achieved by the use of drugs such as cocaine and imipramine to potentiate the pressor effects of the catecholamines by blocking their uptake into the sympathetic neurones. Using this method, Haefely *et al.* (1965) could distinguish between 0.2 and 0.4 ng of noradrenaline.

Rat Uterus

It is well known that the uterus is both relaxed and contracted by adrenaline, depending on the hormonal state of the animal (Axelsson 1971). The uterus of the rabbit and pregnant cat is stimulated by adrenaline, while the uterus of the rat, guinea pig and virgin cat is inhibited. The rat uterus provides one of the most sensitive and specific methods for the determination of adrenaline (Lewis & Waton 1957). Noradrenaline is comparatively inactive; in fact it has only 1/100th-1/200th of the activity of adrenaline. Making use of the fact that adrenaline will inhibit carbachol-induced uterine contraction, as little as 0.1 ng may be detected (Gaddum *et al.* 1949).

Blood Vessels

The blood vessels of the rabbit ear in particular are sensitive to vasoconstrictor drugs. Bioassays relying on the changes in perfusion pressure or in the rate of flow through isolated blood vessels (Schlossmann 1927, de la Lande & Harvey 1965) have appeared. Changes in the diameter of the denervated rabbit ear artery (Armin & Grant 1955) and in the length of a superfused strip of rabbit aorta (Helmer 1961, Bell 1971) are also effective.

In about half of Bell's preparations measurable responses were obtained from 0.1-0.2 ng of noradrenaline. Since adrenaline is also active only total catecholamines may be determined without separation.

Intestinal Muscle

Various intestinal tissues have been found sufficiently reactive to the catecholamines to be employed in bioassays. The rat superfused stomach strip (Armitage & Vane 1964), rabbit intestine (Stewart & Rogoff 1919), chicken rectal caecum (von Euler 1956) and rat colon (Gaddum *et al.* 1949) have all been reported in the literature. The method of Armitage & Vane (1964) relies on catecholamine inhibition of the serotonin-induced contractions of the stomach strip. It is sensitive to 0.2 ng ml^{-1} but does not distinguish between noradrenaline and adrenaline. However the chicken rectal caecum discriminates in favour of adrenaline while the rat colon provides a sensitive test for noradrenaline.

Other Methods

The isolated guinea pig heart (Brooker 1959), the cat nictitating membrane (Gaddum *et al.* 1949) and a whole variety of tissues have been pressed into the service of bioassays over the years. Recently Hiraoka & Serizawa (1974) suggested that the decrease in ear temperature in rabbits and dogs in response to sympathomimetic amines could form the basis of a biological assay. In this case the potency of adrenaline was approximately twice that of noradrenaline.

Effects of Interfering Substances

A major problem with biological assays is that sample extracts often contain substances that effect the assay tissue. Either the interfering substance has a catecholamine-like effect or it potentiates or diminishes the tissue response to catecholamines. The principal active contaminants are serotonin, histamine and acetylcholine. In the intact animal the latter two compounds further bias the results by stimulating adrenal medullary

catecholamine secretion. The rat stomach strip is sensitive to serotonin (Vane 1957, 1964) and the guinea pig ileum is affected by histamine (Vane 1964). Where acetylcholine or histamine are likely to be present the tissue can be treated with atropine sulphate and an anti-histamine such as mepyramine maleate (Schmitterl  w 1951, Eade 1961). Armitage & Vane (1964) overcame the problem of serotonin interference by bathing their rat stomach strip in a $10 \mu\text{g ml}^{-1}$ serotonin solution. This had two advantages. It increased tissue sensitivity to adrenaline and since the tissue was already maximally contracted with serotonin, exogenous serotonin was of less concern. In general however, the presence of interfering substances present in crude extracts or in body fluids is a serious problem. Sample purification (§3.3) is necessary to overcome lack of specificity.

Discrimination Between Catecholamines

If physical separation of noradrenaline and adrenaline is not undertaken the generally employed method for their separate estimation is to assay the same sample with two tissues, either in series or in parallel. The two tissues chosen must have the greatest possible difference in their reactions to noradrenaline and adrenaline. From the known reaction of each tissue to standard mixtures the proportions of each catecholamine may be calculated using simultaneous equations. Discrimination may sometimes be improved with the use of α - and β -blocking agents. A drawback to this approach is that there is no known tissue that has a much greater response to noradrenaline compared to adrenaline, hence it is very difficult to measure the noradrenaline content of samples where it is present in quantities of less than 10% of the total catecholamine content. Suitable tissue combinations include the rat uterus and colon (Gaddum & Lembeck 1949), the rat stomach strip and the chicken rectum (Armitage & Vane 1964), hen rectal caecum and cat blood pressure (von Euler 1949) and the rat uterus with a variety of other responses from the spinal cat blood pressure, frog heart and rabbit duodenum (B  lbring 1949). In the most extensive study of its type

Gaddum *et al.* (1949) used 5 different tissues to distinguish between adrenaline and a range of closely related compounds.

The biological assay reached the peak of its sophistication with the work of Vane (1958, 1964, 1966), who described a blood-bathed organ technique where the circulating blood of the experimental animal was continuously monitored for changes in hormone level. Carotid blood was pumped by a roller pump at a rate of 12 to 15 ml minute⁻¹ over a rat stomach strip and a chick rectum in series. The blood was then collected and returned to the jugular vein (Figure 3-2). The technique has advantages of relative ease with continuous and immediate results. It has been applied to the estimation of the release of catecholamines and angiotensin during haemorrhage (Regoli & Vane 1966), the release of catecholamines induced by peptides such as bradykinin (Staszewska-Barczak & Vane 1965a), histamine (Staszewska-Barczak & Vane 1965b), and nerve stimulation (Marley 1961). The method, while confined to the laboratory, and subject to the difficulties inherent in bioassay is to date the only means of such continuous monitoring of endocrine status.

Thus while bioassays are slow, notoriously difficult to standardize and maintain and are not noted for accuracy, in the hands of experienced physiologists they have made a considerable contribution to our present understanding of catecholamine physiology. Today chemical methods, despite their own limitations, are the methods of choice when absolute levels of catecholamines are measured.

3.5 COLORIMETRIC ASSAYS

The earliest methods for the chemical estimation of catecholamines were colorimetric (Shaw 1938, von Euler & Hamberg 1949, Oesterling & Tse 1961). The methods of Shaw (1938) and James & Kilbey (1951) made use of the reducing properties of catecholamines with an easily measured indicator. The complete lack of specificity makes prior purification, and if required, separation, mandatory. In the procedure of Shaw (1938) adrenaline was first separated

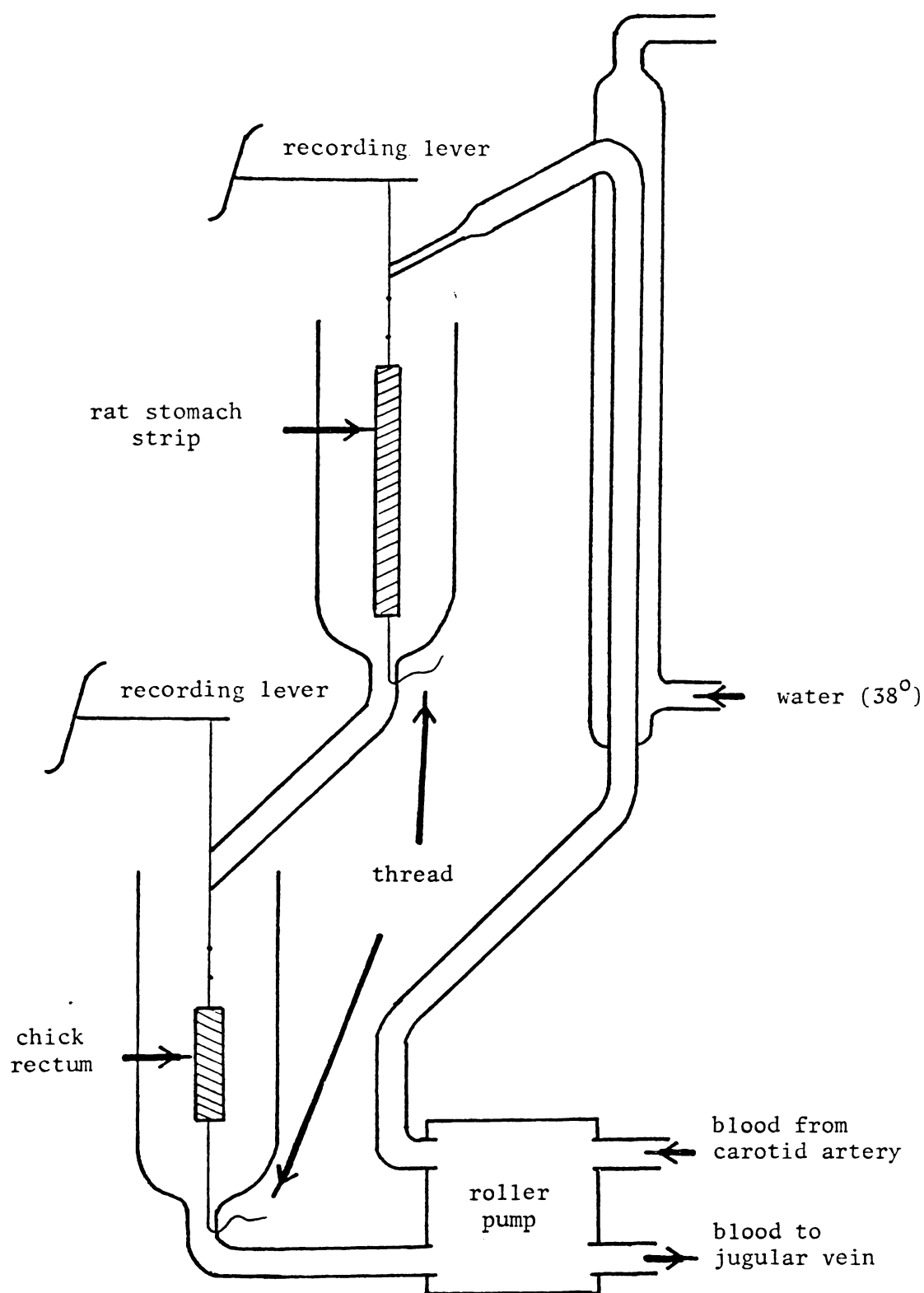


Figure 3-2. Continuous catecholamine bioassay with isolated blood-bathed organs. [from Vane 1964]

with aluminium oxide and then reacted with arsenomolybdic acid; paper chromatography in conjunction with arsenophosphotungstic acid was used by James & Kilbey (1951).

A solution containing a single catecholamine in a concentration higher than $10 \mu\text{g ml}^{-1}$ can be easily assayed with ultraviolet adsorption at 279 nm. Visible adsorption can be used if the catecholamine is first oxidized to the red coloured chrome derivative (von Euler 1956). With iodine oxidation a mixture of chromes and iodochromes is formed (Figure 3-3). A differential assay is possible by oxidizing two aliquots at pH 4 and pH 6 (von Euler & Hamberg 1949). Under these conditions <10% of the noradrenaline is oxidized at pH 4 in 2 minutes while total catecholamine oxidation is accomplished at pH 6. A blank is prepared without iodine and the colours developed are measured at 529 nm. Using simultaneous equations the amounts of each amine may then be determined, though if either catecholamine is present in amounts <5% the error can be considerable. A similar method was developed by Suzuki & Ozaki (1951) substituting permanganate for iodine and stopping the reaction with hydrogen peroxide rather than with the thiosulphate; reading at 539 nm. Heating at 100° with concentrated nitric acid followed by treatment with alkali gives rise to coloured nitrated products with an adsorption maximum of 380 nm (Hassan & Zaki 1976). The method is suitable for concentrations in the $\mu\text{g ml}^{-1}$ range.

The comparatively low sensitivity of colorimetric methods makes them unsuitable for catecholamine assay excepting pharmaceutical preparations or adrenal medulla extracts.

3.6 FLUORIMETRIC ASSAYS

Fluorimetric methods for the assay of catecholamines are more specific than either biological or colorimetric assays. On the one hand their reproducibility is greater than biological assays and on the other they are more sensitive than colorimetric assays. The catecholamines have a native nonspecific fluorescence due to their phenolic structure (excitation wavelength 285 nm, emission

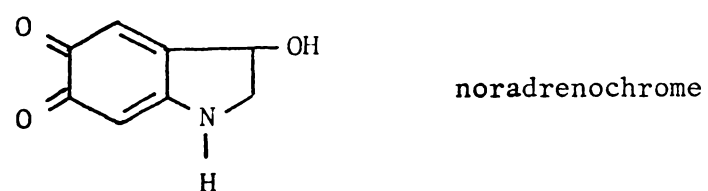
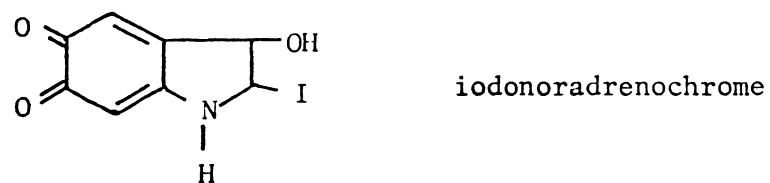
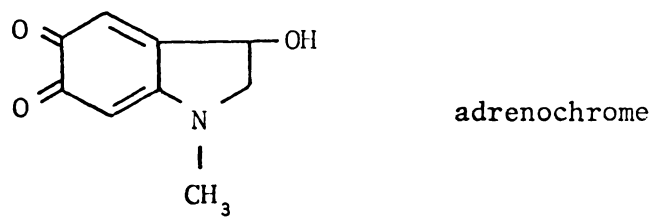
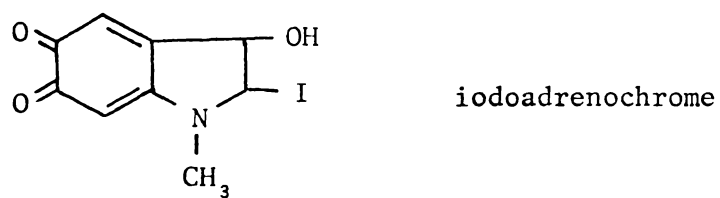


Figure 3-3. Oxidation products of adrenaline and noradrenaline with iodine.

wavelength 325 nm) (Duggan *et al.* 1957). An increase in the discrimination between catecholamines and an increase in the sensitivity by a factor of about 20 is possible by their conversion to fluorescent derivatives. Two principal methods of accomplishing the conversion have been developed: the ethylenediamine condensation method (EDA) and the trihydroxyindole method (THI). A third method recently reported, but little used in biological studies as yet, involves catecholamine condensation with fluorescamine. Most catecholamine metabolites may also be assayed fluorimetrically (Udenfriend 1969, Weil-Malherbe 1971, Nagatsu 1973).

Instrumentation

Fluorimetric methods have been greatly improved over the last 25 years, not only because of improvements in techniques but also due to advances in commercial fluorimeters (Udenfriend 1969). The early filter fluorimeters though adequate for urinary catecholamines, were noisy and lacked specificity in comparison to the modern monochromometer instruments. Careful attention must also be paid to grating types and beam slit-widths for the optimum compromise between sensitivity and specificity. The choice of photomultipliers which can differ markedly in their responses over the same spectral range is also important to assay sensitivity. In the analysis of plasma catecholamines the limiting factor has been the small sample fluorescence compared to the blank which results in the fluorimeter being stretched to the limits of its sensitivity. Several manufacturers offer an off-axis ellipsoidal mirror condensing system which, by increasing the intensity of the light incident on the sample increases the fluorescence signal - though of course the blank is similarly increased. At maximum signal amplification lamp noise level is often sufficient to prevent accurate analysis of resting plasma levels. A number of devices are now available to reduce the noise level. Some fluorimeters have a double beam, while others incorporate an electromagnetic arc stabilizer or a ratio photometer. In general one of these types is essential for successful plasma analysis.

Ethylenediamine Condensation Method

Catechol compounds may be converted into highly fluorescent and relatively stable compounds by oxidation and condensation with ethylenediamine (Natelson *et al.* 1949). Noradrenaline reacts with two reagent molecules and loses its sidechain to form 1,2,3,4-tetrahydro-1,4,5,8-tetraaza-anthracene, while adrenaline reacts with one molecule of ethylenediamine and retains the N-methyl-ethanolamine sidechain forming 2,3-dihydro-3-hydroxy-1-methyl-pyrrolo-[4,5:g]-quinoxaline (Harley-Mason & Laird 1958, Weil-Malherbe 1960) (Figure 3-4). A number of catechol compounds, including catechol itself, form the same product with ethylenediamine as noradrenaline. The elimination of the β -ethanolamine sidechain depends on the presence of the β -hydroxy group.

In the procedure of Mangan & Mason (1958) based on that of Weil-Malherbe & Bone (1952), the catecholamines are isolated by aluminium oxide chromatography; 0.5 ml of ethylenediamine hydrochloride is added followed immediately by 0.7 ml of ethylenediamine. After 20 minutes at 50° the samples are cooled and saturated with sodium chloride. Extraction of the fluorescent products is carried out with 6 ml of isobutanol, shaking for 4 minutes on a mechanical shaker. With the excitation wavelength at 420 nm the fluorescence due to the condensation product of adrenaline at 580 nm is twice that of the noradrenaline product, while at 510 nm the position is reversed. Hence it is possible to distinguish between noradrenaline and adrenaline. However at 580 nm the fluorescence intensity of both compounds is barely 10-20% of their maximum (at 540 nm), limiting the sensitivity of the differential assay.

The major criticism levelled against this method is that it lacks specificity (Valk & Price 1956, Price *et al.* 1960, Siegal *et al.* 1961). Such criticism is based partly on theoretical grounds regarding the non-specificity of the reaction and partly on the finding that levels of plasma catecholamines in subjects at rest measured by this technique result in higher values than when the THI procedure is employed. Both dopamine (which is present in

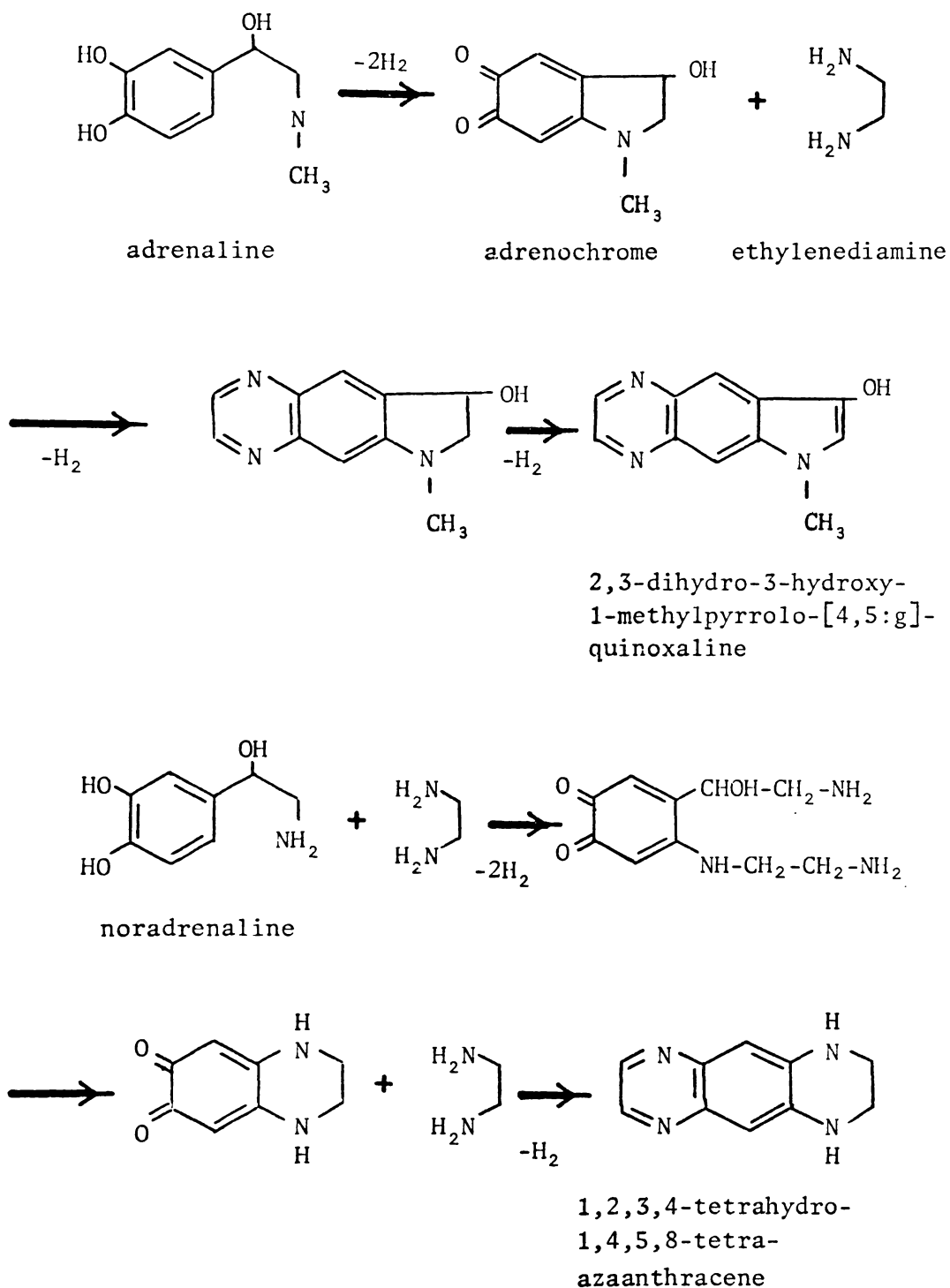


Figure 3-4. Reaction of ethylenediamine with adrenaline and noradrenaline.

plasma) and 3,4-dihydroxyphenylacetic acid, for example, form fluorescent products (Valk & Price 1956). The condensation products of acid catechols including 1-dopa are excluded during the isobutanol extraction. The plasma levels of catecholamines determined by this assay were initially very high - ranging up to 20 ng ml⁻¹ (Weil-Malherbe & Bone 1952, 1953, 1954, 1957c). While estimates have been progressively reduced with refinements in the methodology, reported plasma values are still above accepted concentrations reported by other procedures (Table 5-1) (Manger *et al.* 1969). While the EDA method has excellent sensitivity it has given way since 1960 to the more specific, though more demanding, THI procedure. Nonetheless the reaction, in conjunction with chromatographic separation, is suitable for the estimation of small quantities of catecholamines. After extraction in dichloromethane the acetylated derivatives of the three catecholamines can be separated by paper chromatography and then condensed with ethylenediamine (Lavery & Sharman 1965).

Trihydroxyindole Method

The THI method has developed from an observation of Loewi in 1918 who found that adrenaline in alkaline solution gave rise to a substance which emitted a strong yellow-green fluorescence. Gaddum & Schild (1934) attempted to apply the principle to adrenaline in blood but the presence of interfering substances and the rapid decay of the fluorescence defeated their efforts. The modern THI estimation of catecholamines is based on their transformation to highly fluorescent 3,5,6-trihydroxyindoles or lutines (Ehrlén 1948, Bu'Lock & Harley-Mason 1951). The essence of the assay is an initial oxidation to a quinone which cyclises to form, in the case of noradrenaline, noradrenochrome. In the presence of alkali, noradrenochrome rearranges to form the fluorescent noradrenolutin (Figure 3-5). Unless this compound is stabilized with a reducing agent (such as ascorbic acid or sodium sulphite) it undergoes further oxidation to a non-fluorescent product.

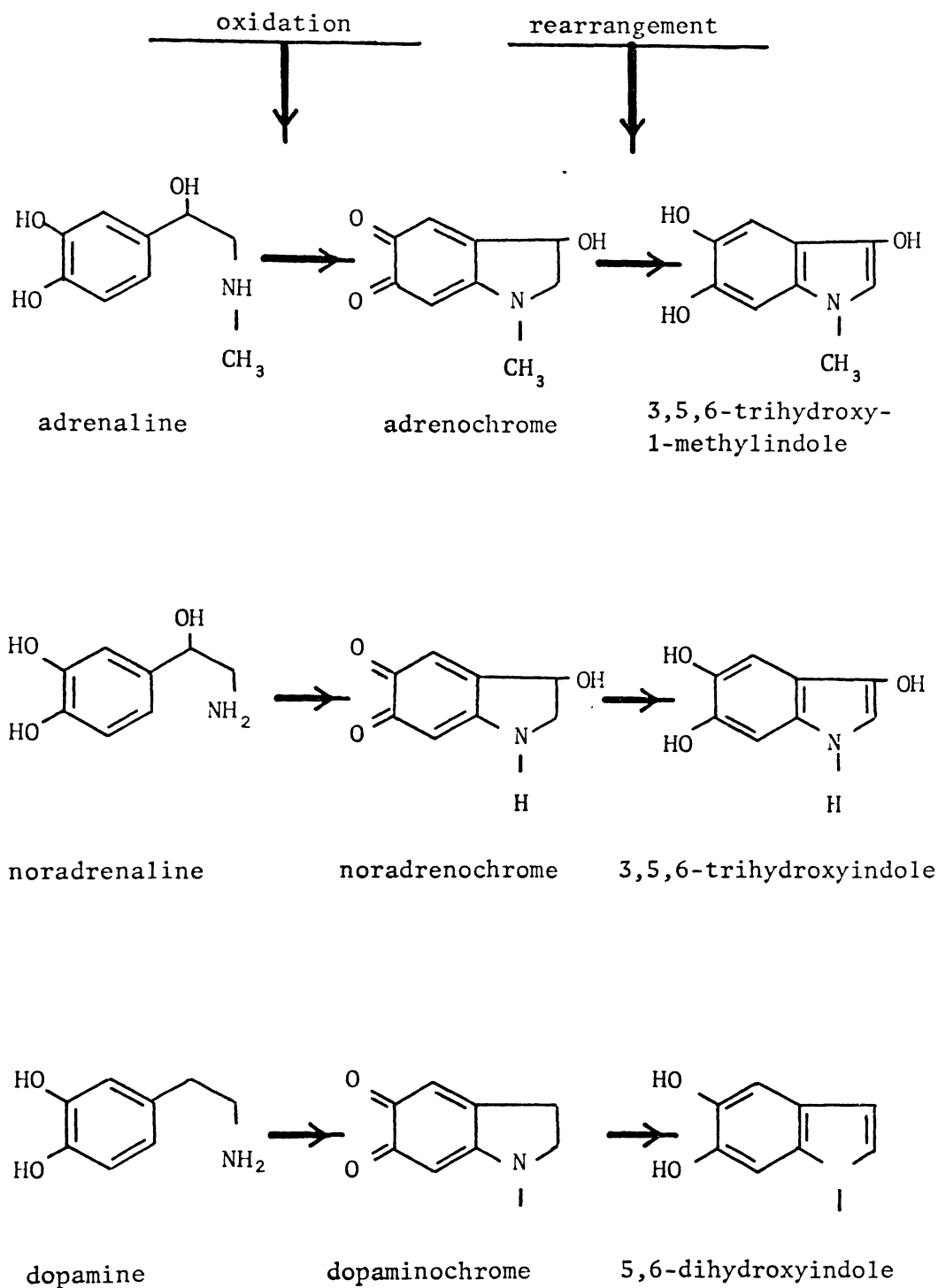


Figure 3-5. Oxidation and rearrangement of catecholamines to fluorescent trihydroxy or dihydroxyindoles.

The plethora of published procedures for the THI assay of catecholamines is an indication of the difficulty that is encountered in measuring minute quantities. Some of the potential random and systematic errors will be considered as each step in the THI method is discussed. The oxidation step has been carried out using iodine, sodium periodate, manganese dioxide, potassium permanganate, sodium hypochlorite, hydrogen peroxide and potassium ferricyanide and, to a lesser extent, manganese dioxide. An advantage of potassium ferricyanide is that dopamine, normetanephrine and metanephrine, if present, will not interfere with the assay of noradrenaline and adrenaline (Häggendal 1966). Further, it has the advantage that isomerization occurs within 4 minutes while with iodine the process can take an hour or longer, depending on the amine (Crout 1961b, Häggendal 1963a, Lavery & Taylor 1968). In the differential assay of the catecholamines use is made of the fact that adrenaline is readily oxidized at a pH as low as 3 whereas the oxidation of noradrenaline is very slow - with the position being reversed at pH 6 (§3.5). Lavery & Taylor (1968) made an extensive study of this phenomenon using iodine oxidation and their results are presented in Figure 3-6a. Of iodine, ferricyanide and manganese dioxide, the best differentiation between noradrenaline and adrenaline is achieved by iodine oxidation. The choice of buffer is also important in the intensity of the final fluorescence (Lavery & Taylor 1968, Weil-Malherbe & Bigelow 1968). A 10-fold improvement in noradrenaline/adrenaline discrimination is claimed by Kahane & Vestergaard (1965) using a sodium phosphate/metaphosphate buffer at pH 2.85 in preference to the more usual acetate buffer at pH 3.5.

In general oxidation is interrupted and the chrome derivatives are converted into fluorescent lutines by the addition of a mixture of ascorbic acid (to reduce excess oxidizing agent and to stabilize the lutines) and sodium hydroxide for tautomerization. It is well recognized however, that ascorbic acid itself may form fluorescent oxidation products, especially when ferricyanide is used as the oxidizing agent. This results in high and rising

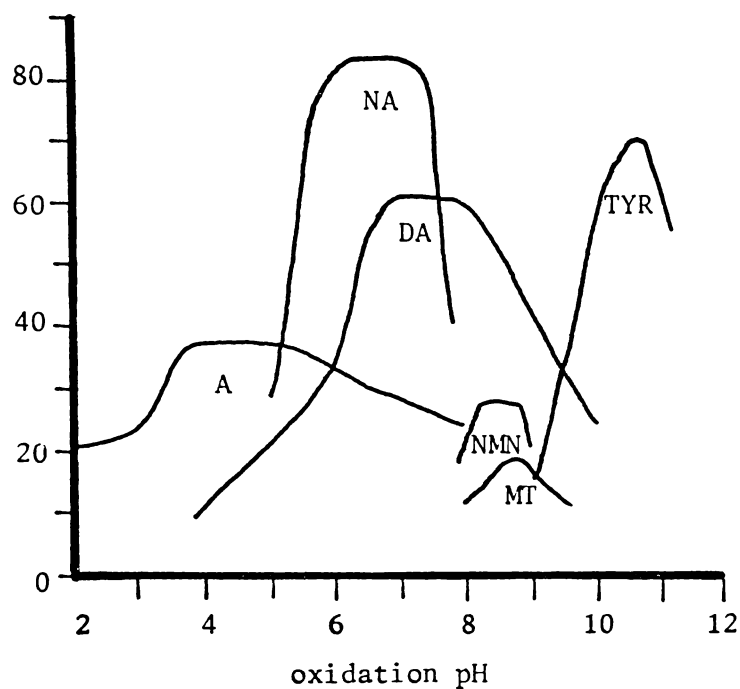


Figure 3-6a. Amount of fluorescence produced at different oxidation pH's in the trihydroxyindole assay.

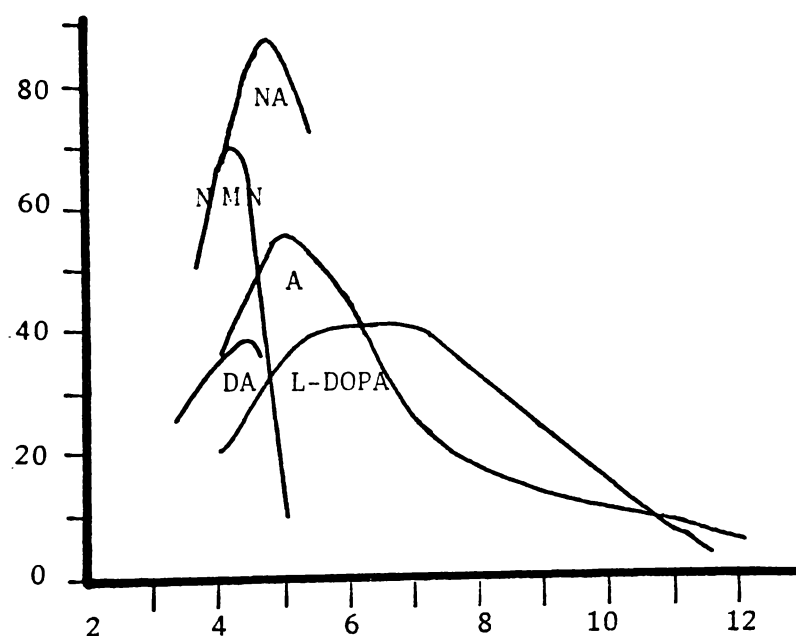


Figure 3-6b. Effect of final pH of final solution on fluorescence intensity developed after oxidation and alkaline re-arrangement in the trihydroxyindole assay.

[from Laverty & Taylor 1968]

blanks (Crout 1959, Gerst *et al.* 1966). The increase in fluorescence owing to ascorbic acid decomposition can be reduced by the addition of a second reducing agent such as ethylenediamine or propylenediamine (von Euler & Lishajko 1961b), thioglycollic acid (Merrills 1963), β -thiopropionic acid (Palmer 1964, Martin & Harrison 1968), or sodium borohydride (Gerst *et al.* 1966). Vaage (1974) approached the problem by first reducing the excess ferricyanide with an exactly equivalent amount of ascorbic acid then stabilizing adrenochromes with dithiothreitol during tautomerization at pH 13. Other authors have replaced ascorbic acid by 2,3-dimercaptopropanol (Häggendal 1963a), mercaptoethanol-sodium sulphite (Weil-Malherbe & Bigelow 1968) and mercaptoethanol-formaldehyde (Valori *et al.* 1970), all claiming reduced blanks and increased fluorescence.

Most analysts using ascorbic acid stipulate that it should be mixed with the sodium hydroxide before addition since this appears to increase the catecholamine fluorescence. At pH 3 the effect appears especially marked. In acid solution ascorbic acid exists largely in the ketohydroxy configuration but on the addition of alkali the more strongly reducing enediol configuration is favoured. When thiol compounds are used as reducing agents the results are identical whether the solutions are added separately or as a mixture.

Weil-Malherbe & Bigelow (1968) found that the addition of cupric ions at an oxidation pH of 3 had the effect of increasing the fluorescence about 10-fold, in the presence of mercaptoethanol as the reducing agent. No effect was found at pH 6 and seventeen other metal ions were without effect.

An alternative approach to the problem of fluorophore instability is to reduce the excess oxidizing agent and, after a short period for rearrangement in alkaline solution, to reacidify the solution to about pH 5-6 (Price & Price 1957, Carlsson & Waldeck 1958, Laverty & Taylor 1968). Such a procedure permits the use of sodium sulphite as the reducing agent with no decomposition problems. Laverty & Taylor (1968) found that all the amines

tested required 5 minutes exposure to alkaline conditions for tautomerization to occur, excepting adrenaline and metanephrine where the requirement was 1 minute. Acidification of the alkaline reaction mixture using glacial acetic acid resulted in increased final fluorescence with all compounds (Figure 3-6b). The different pH maxima may also be used to increase discrimination.

There is some uncertainty as to the best method of preparing a sample blank. A faded blank is made by delaying or omitting the addition of the reducing agent after the sodium hydroxide is added. During this time the lutines are decomposed (Lund 1950). Alternatively some workers prefer non-oxidized blanks by reversing the order of the oxidizing and reducing agents (Price & Price 1957) or omission of the oxidizing agent (Diamant & Byers 1975). However, neither of these procedures is completely satisfactory owing to incomplete lutein decomposition in the first case and spontaneous oxidation in the second. O'Hanlon *et al.* (1970) found that both blanks were unstable, increasing with time, variable and dependent on the total catecholamine content. They therefore used a third type in which the sample was added last to the reagent mixture. This blank, it was claimed, was stable, consistent and never more than 5% greater than the external acid blank. Vaage (1974) introduced a new type of blank by oxidizing the trihydroxyindoles with hydrogen peroxide to the corresponding non-fluorescent quinones.

A number of authors have emphasized the importance of high quality water and pure reagents to successful fluorimetric assay. Double or triple distilled water is normally used. Deionized water should be checked to ensure that fluorescent contaminants are not leached from the resin. Ground glass joints without lubricants and borosilicate glass should be used throughout the assay to ensure the lowest possible blanks.

If prior chromatographic separation of the catecholamines (Crawford & Law 1958) is not undertaken the following steps may be

taken to increase discrimination between dopamine, noradrenaline and adrenaline:

1. Different oxidation pH (Anton & Sayre 1962, Laverty & Taylor 1968, Griffiths *et al.* 1970).
2. Different final pH after reacidification (Laverty & Taylor 1968, Griffiths *et al.* 1970).
3. A longer time interval between the addition of alkali and reacidification discriminates in favour of noradrenaline since the adrenolutin develops and decomposes more rapidly than noradrenolutin (Laverty & Taylor 1968, Griffiths *et al.* 1970).
4. The use of alkaline thioglycolate stabilizer protects only noradrenolutin (Merrills 1963, Mabry & Warth 1969, Hanig *et al.* 1972).
5. Exposure to ultraviolet light destroys noradrenolutin but has little effect on adrenolutin (Hanig *et al.* 1972).
6. The use of fluorimeter optical discrimination (noradrenaline: 380/480 nm, adrenaline: 425/500 nm - Griffiths *et al.* 1970).

Usually one or a combination of these procedures enables a satisfactory resolution between noradrenaline and adrenaline. The residual mutual interference can be taken into account using simultaneous equations. Hanig *et al.* (1972) reported interference of less than 1% in the assay of adrenaline by the presence of an equimolar amount of noradrenaline. The interference by adrenaline in the noradrenaline assay was 4%.

L-dopa and dopamine may also be determined by the THI procedure, though the fluorescence is slow to develop (Carlsson & Waldeck 1958). A variety of oxidizing agents used includes sodium periodate (Anton & Sayre 1964), iodine (Laverty & Taylor 1968, Tyce *et al.* 1970, Fahn *et al.* 1972, Johnson *et al.* 1973) and potassium ferricyanide (Curzon *et al.* 1972, Attack 1973, Prasad *et al.* 1975). The latter oxidizing agent had the effect of

reducing interference by 3-O-methyldopa (the major metabolite of l-dopa) in the assay of Curzon *et al.* (1972). It has been noted that the presence of dipotassium hydrogen phosphate in the buffer during oxidation produces a curvilinear relationship between concentration of dopamine and fluorescence intensity (Greenland & Michaelson 1974). Laverty & Taylor (1968) recommend heating at 100° for 40 minutes as the final step to accelerate fluorophore development. The same effect may be accomplished by ultraviolet light (Atack 1973, Johnson *et al.* 1973). Because of the comparatively low reactivity and the different excitation/emission fluorescence wavelengths of l-dopa (330-380 nm) and dopamine (320-375 nm) derivatives interference by noradrenaline and adrenaline in the assay is negligible. Nonetheless in most published procedures there is considerable mutual interference between l-dopa and dopamine. Under most circumstances with biological samples l-dopa is present in insignificant amounts. Where l-dopa is administered as a drug chromatographic separation is usually undertaken (Tyce *et al.* 1970, Spiegel & Christian 1971, Fahn *et al.* 1972, Prasad *et al.* 1975). The modification of Atack (1973) in the assay of dopamine involves both the oxidation by ferricyanide and the tautomerization, in an alkaline solution in the presence of sodium metabisulphite. The ferricyanide is reduced comparatively slowly by the metabisulphite over 30 seconds while dopamine oxidation occurs rapidly. Since neither the oxidation pH nor the timing is critical, large numbers of samples can be assayed simultaneously. The use of optical (Hare & Vogel 1970) or native fluorescence (Hare *et al.* 1974) detection after separation by ion exchange chromatography has also been used for the determination of l-dopa and its principal metabolites in plasma and urine.

The work of Laverty & Taylor (1968) on the parameters affecting each stage of the THI procedure was of great importance since the most sensitive assays of plasma catecholamines are based on their conclusions. Iodine was chosen as the oxidant since it reacts most efficiently with a wide range of catechol compounds. In outline the general method adopted was as follows (see Table 3-2

for volumes of alkaline sulphite and acetic acid solutions required for different compounds):

1. 0.1-0.5 ml of sample is made up to 1.1 ml with the appropriate buffer.
2. Iodine (0.05 ml of 0.02N I_2 in NaI 5% w/v) is added and the solution stirred.
3. After 3 minutes alkaline sulphite solution is added ($Na_2SO_3 \cdot 7H_2O$ 2.5% w/v, Na_2 EDTA 1% w/v in 2.5N NaOH).
4. Depending on the measured compound, an appropriate volume of glacial acetic acid is added after either 1 or 5 minutes.
5. After a variable time period, again depending on the compound, with heating if necessary, the sample fluorescence is read in the fluorimeter at the wavelength maximum.
6. Samples with added internal standard, and completely reversed blanks are run in parallel with the unknown sample.

In Table 3-2 a summary of the optimum conditions for the catecholamines and a number of related substances is presented. Several sensitive plasma assays (Griffiths *et al.* 1970, O'Hanlon *et al.* 1970, Diamant & Byers 1975) and a tissue assay (Waldmeier *et al.* 1974) based on the data of Lavery & Taylor (1968) have appeared in the literature. O'Hanlon *et al.* (1970) increased the assay sensitivity by a reduction of the aluminium oxide eluent volume to 1.2 ml (compared to the usual 4 ml or more) and increasing adrenaline recovery to 97%. Diamant & Byers (1975) introduced the use of tritiated catecholamines to give a recovery value for each sample.

The values obtained for the resting levels of plasma catecholamines by these assays correspond closely to those found using the sensitive radioenzymatic assays (§3.10). Furthermore, several physiological experiments involving plasma catecholamine measure-

Table 3-2

Optimal Conditions for Determination of Catecholamines and Related Compounds
by the Trihydroxyindole Method

Compound	Oxidation		Tautomerization		Acidification		Development		Wavelengths	
	Buffer	pH	Sulphite (ml)	Time (min)	Acid (ml)	Final pH	Time (min)	Temp °	Excit.	Emission (nm)
L-dopa	citrate	5.4	0.25	5	0.05	5.7	40	100	330	380
DA	phosphate	7.0	0.25	5	0.10	4.4	40	100	320	375
NA	phosphate	6.5	0.5	5	0.14	4.8	25	20	380	480
A	citrate	4.0	0.5	1	0.10	5.0	0	20	410	500
NMN	borate	8.6	0.5	5	0.30	4.2	25	20	380	475
MN	borate	9.1	0.5	1	0.10	5.6	0	20	410	490
Octopamine	carbonate	9.0	0.4	5	0.20	4.7	30	100	375	475
Tyramine	carbonate	9.9	0.4	5	0.15	4.5	40	100	320	375
Tyrosine	carbonate	10.6	0.4	5	0.10	5.0	40	100	330	380

[from Laverty & Taylor 1968]

ment by this variation of the THI procedure have been published (Bannister & Griffiths 1972, Wilkerson *et al.* 1974) enhancing confidence in its practicality.

The application of the principles of automated chemical analysis ensures the accuracy and reproducibility of each step and permits a considerable increase in the number of samples processed, though at the cost of a loss in sensitivity (Fiorica 1965, Martin & Harrison 1968, Hanig *et al.* 1972, Peyrin & Cottet-Emard 1973, Andersson *et al.* 1974, Waldmeier *et al.* 1974). Automated assay also allows better temperature control which is important to accurate fluorescence measurements. Andersson *et al.* (1974) found that the fluorescence at 10° is about 40% greater than at 22° in urinary samples. However the very low concentrations found in plasma have prevented the adaption of automated flow chemistry to plasma catecholamine extracts although clinical screening for some sympathoadrenal conditions may be a practical possibility (Wood & Mainwaring-Burton 1975).

Determination of Primary Amines with Fluorescamine

With the discovery of a highly fluorescent compound formed by the condensation of primary amines with ninhydrin and phenylacetaldehyde (Samejima *et al.* 1971) and its structural determination (Weigle *et al.* 1972), a new reagent [4-phenylspire[furan-2(3H)-phthalan]-3,3'-dione(fluorescamine)] has been synthesized which reacts with primary amines to yield highly fluorescent ternary products (Figure 3-7). The reaction is simple in comparison to the EDA and THI methods since, at pH 9, reaction with primary amines proceeds at room temperature with a half time of a fraction of a second. Excess reagent is decomposed by hydrolysis with a half time of several seconds (Udenfriend *et al.* 1972). Fluorescamine, as well as its hydrolysis products, is non-fluorescent and sensitivity with many amines is in the picomole range. Separation and quantitation of the reaction products of dopamine and noradrenaline and of 3-methoxytyramine and normetanephrine have been accomplished by high pressure liquid chromatography on a gel column (Imai 1975). Since the dopamine and noradrenaline

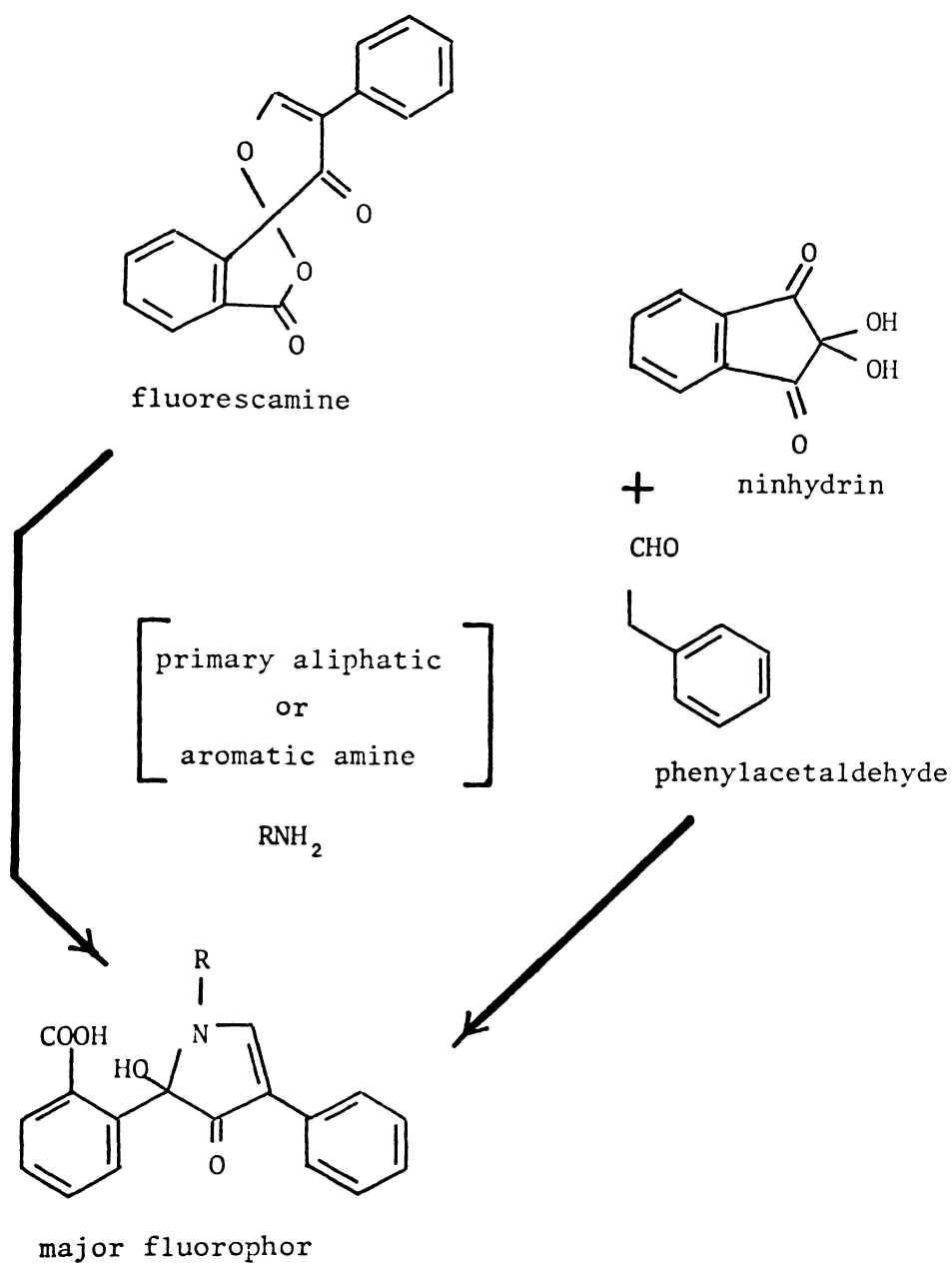


Figure 3-7. Chemical reaction of fluorescamine with primary amino compounds and the alternative pathway with ninhydrin and phenylacetaldehyde.

fluorophores can be chelated by borate and eluted rapidly from the column it is possible to determine 3-methoxytyramine and normetanephrine even in the presence of large quantities of the parent compounds. Sensitivity approaches that of the THI method. Other reports include the assay of 3-methoxy-4-hydroxy-phenylalanine (Cheng *et al.* 1975) and 1-dopa and dopamine (de Silva & Strojny 1975) though in the latter report sensitivity is not high (twice blank approximately $1 \mu\text{g ml}^{-1}$).

Thus while no applications to plasma catecholamines have appeared this method would appear to have some potential for the assay of dopamine and noradrenaline in conjunction with a suitable separation technique.

3.7 RESONANCE RAMAN ASSAYS

The use of resonance Raman spectroscopy has been advocated by Rahaman & Morris (1976) as a means of overcoming the quenching problems and lack of differentiation between noradrenaline and adrenaline encountered with fluorimetry. However the sensitivity indicated in the report ($\mu\text{g ml}^{-1}$ range) would limit its applicability to concentrated biological samples. Further developments in instrumentation may increase the potential of this approach.

3.8 LIQUID CHROMATOGRAPHIC ASSAYS

The problems of assaying simultaneously the different catecholamines, their metabolites and closely related compounds in a single biological sample are formidable. For example Armstrong *et al.* (1956) found 49 simple phenolic acids in human urine of which 23 were identified. The answer to the problems of specificity lies in chromatography, but the techniques of paper and thin layer chromatography pose problems in accurate quantitation and sensitivity. Hence numerous attempts have been made to develop more sensitive chromatographic procedures.

Considerable progress has been seen in column liquid chromatography over the last few years through the introduction of techniques to pack columns efficiently with particles of small diameter and the use of high pressures for rapid throughput of samples. The stationary phase in most catecholamine assays is usually a strong cation-exchange resin (Kojima-Sudo 1974, Mori 1974, Kissinger *et al.* 1975, Keller *et al.* 1976, Riggin *et al.* 1976) although for separation of 3-methoxy-4-hydroxyphenylacetic acid and 3-methoxy-4-hydroxymandelic acid the use of a gel composed of particles of styrene-divinylbenzene is noted (Yoshida *et al.* 1976). Kojima-Sudo (1974) used a gradient concentration elution to effect better separation between catecholamines. In all procedures prior purification with aluminium oxide is a necessary prerequisite. Ion partition chromatography, relying on the extraction of the ionized aqueous catecholamine into an organic solvent by the formation of an ion pair with an oppositely charged species, has been developed to the point where 6 catecholamines and metabolites can be quantitatively separated in only 4 minutes (Persson & Karger 1974).

With chromatographic separation several non-specific means of detection are possible including ultraviolet adsorption (Yoshida *et al.* 1976), automated fluorimetric analysis (Kojima-Sudo 1974, Mori 1974) and electrochemical detection (Kissinger *et al.* 1975, Keller *et al.* 1976, Riggin *et al.* 1976). Electrochemical detection is based on the loss of 2 electrons by the eluted catecholamines at an electrode surface of the appropriate potential. Oxidation to the corresponding quinones occurs. Given a constant flow rate and temperature the current detected across the electrodes is proportional to the concentration of the oxidized compound. This technique appears to have the potential to measure plasma levels of the catecholamines into the picogram range (Keller *et al.* 1976). Nonetheless the authors emphasize, as with fluorimetric and radioenzymatic procedures, that careful attention to detail is essential. Elution time is 20 minutes.

3.9 GAS-LIQUID CHROMATOGRAPHIC AND MASS FRAGMENTOGRAPHIC ASSAYS

Since the catecholamines and their metabolites are highly polar and possess poor volatility early attempts to chromatograph catecholamines in the free state resulted in asymmetric peaks exhibiting considerable tailing and erratic results owing to decomposition (Fales & Pisano 1962, Parker *et al.* 1962).

Derivative formation is therefore necessary for any gas-liquid chromatographic analysis, and, when electron capture detection is utilized, serves the additional function of producing a compound to which the detector can sensitively respond. The derivation methods most often used for hydrogen-flame or argon-ionization detectors are formation of a trimethylsilyl ether (Sen & McGeer 1963, Kawai *et al.* 1966, Moffat & Horning 1970a, Haeffner *et al.* 1976) or acetylation (Brooks & Horning 1964, Frère & Verley 1967). Dopamine, noradrenaline, tyramine, normetanephrine and metanephrine in urine can be analyzed as their trifluoroacetyl-trimethylsilyl derivatives or as trimethylsilyl derivatives on polyamide columns (Haeffner *et al.* 1976), while the methane and n-butaneboronates are suitable derivatives for the detection of urinary 3-methoxy-4-hydroxyphenylglycol (Cagnasso & Biondi 1976). The most sensitive assay using hydrogen-flame detection is that of Lovelady & Foster (1975). As little as 0.1 pg of either noradrenaline or adrenaline, converted to their trifluoroacetic acid derivatives in 2-3 μ l of injected fluid was detectable; corresponding to low physiological levels in plasma. Coefficients of variation of 3.97% and 6.14% were obtained for 3 replicate determinations of standard noradrenaline and adrenaline solutions on 3 separate days. On the whole however these derivative methods lack sufficient sensitivity for anything but fairly high catecholamine levels.

The possibility of considerably increasing the sensitivity of GLC determination in combination with electron capture detection has been studied in several laboratories during the last few years. Electron capture detection, under certain conditions, is as much as 2000 times as sensitive as flame ionization. A number of halogenated reagents has been found suitable for catecholamine

derivation: trifluoroacetic anhydride (Clarke *et al.* 1967, Kawai & Tamura 1968, Änggård & Sedvall 1969), pentafluoropropionic or heptafluorobutyric anhydride (Änggård & Sedvall 1969), N-heptafluorobutyryl imidazole (Cummins & Fourier 1969), pentafluorobenzaldehyde (Moffat & Horning 1970a,b) and 2,4-dinitrofluorobenzene (Walle 1968). It should be born in mind that the electron capture detector gives a response that is unique for each molecular structure and as such is not suited to the simultaneous determination of a large number of compounds; its sensitivity is subject to a large number of factors and its linear response range is not usually great. Another problem mentioned by some (Horning *et al.* 1967, Haeffner *et al.* 1976) is the simultaneous occurrence of a number of derivatives, perhaps not unexpectedly with the multifunctional catecholamine molecules. Moffat & Horning (1970a,b), on the other hand, claim that as little as 10 pg of dopamine or noradrenaline could be measured after conversion to the pentafluorobenzaldehyde derivative. Methods for urinary dopamine, noradrenaline and adrenaline (Wong *et al.* 1973), 3-methoxy-4-hydroxyphenylacetic acid and 3-hydroxy-4-methoxyphenylacetic acid (Dziedzic *et al.* 1973), 3,4-dihydroxyphenylacetic acid (Weg *et al.* 1975) and 3-methoxy-4-hydroxyphenylglycol (Fellows *et al.* 1975) have appeared. Elimination of the mutual interference between dopamine and l-dopa, a limiting aspect of most fluorimetric assays, is achieved by gas chromatographic determination of their pentafluoropropionic derivatives (Mizuno 1977). The method is suitable for monitoring plasma levels of these compounds in patients on l-dopa therapy. Even plasma 3-methoxytyramine, normetanephrine and metanephrine have been detected as their trifluoroacetates (Wang *et al.* 1974, 1975b). Care to prevent column contamination by impurities in solvents or from crude preparations of commonly used hydrolytic enzymes and the necessity for cleanliness have been stressed (Goodwin *et al.* 1972).

The most recent advances, extending sensitivity by a further 1000-fold, have been to use a mass spectrograph in series with the gas chromatograph. The connection is made via a molecular separator

which allows 50% of the molecules heavier than helium to enter the ion-source of the mass spectrograph while removing 99% of the carrier gas from the system. In this way the carrier gas does not interfere with the mass spectral analysis (Cattabeni *et al.* 1972). As each compound is flushed from the column the mass spectrograph is focused onto its most characteristic mass number. Thus for 2 compounds to interfere with each other, identical column retention times and the formation of fragments of identical m/e ratios must occur. Background interference is generally less than for gas-liquid chromatography alone. With this technique quantities in the picomole range can be measured and it is possible to identify as little as 3 femtomoles of a substance whose fragmentation pattern is established (Karoum *et al.* 1972, Koslow *et al.* 1972). With internal standards labelled with stable isotopes, such as deuterium, sensitivity may be even further increased.

At an increasing rate reports are now appearing on the application of mass spectrometry to the measurement of biogenic amines in medicine and neurology in particular. Sjöquist and colleagues have developed an assay for 3-methoxy-4-hydroxyphenylacetic acid (homovanillic acid) using the deuterium labelled analogue as an internal standard and by conversion to the heptafluorobutyryl methyl ester (Sjöquist & Ånggård⁰ 1972, Sjöquist *et al.* 1973). Homovanillic acid was measurable in cerebrospinal fluid, plasma and urine as well as in the mouse brain. The precision of this assay has been notably extended by Fri *et al.* (1974) by the use of pentafluoropropanol in pentafluoropropionic anhydride to effect derivatization and improved instrumentation. The procedure is capable of determining serotonin (5-hydroxyindoleacetic acid) as well as homovanillic acid in cerebrospinal fluid with an experimental error (SD/mean) of 7% or less and a recovery of added standards of 93-96%.

Sjöquist *et al.* (1975) have extended their mass spectral assays to include 3-methoxy-4-hydroxyphenylglycol (MHPG) after conversion to the trifluoroacetyl derivative. The results in Table 3-3

of levels of MHPG in various tissues and body fluids are an indication of the sensitivity and precision of mass spectrometry.

Table 3-3
Levels of MHPG in Tissues and Body Fluids
from Humans and Rats ($\pm$ SD)

Fluid or Tissue	MHPG Level (ng/ml) or (ng g ⁻¹)	No. of Analyses	Coefficient of Variation (%)
Human plasma	10.3 $\pm$ 0.6	18	5.4
Human urine	1363.1 $\pm$ 38.7	20	2.8
Human CSF	13.4 $\pm$ 1.1	16	8.2
Rat urine	4420.8 $\pm$ 158.4	6	3.6
Rat brain	47.9 $\pm$ 3.0	10	6.2
Rat liver	18.2 $\pm$ 2.4	6	13.0

[from Sjöquist *et al.* 1975]

Other groups have measured β -phenylethylamine in various tissues (Boulton *et al.* 1973); dopamine, noradrenaline, serotonin and acetylcholine in rat brain tissue (Koslow *et al.* 1974); adrenaline in brain tissue (Koslow & Schlumpf 1974); dopamine and noradrenaline in rat adrenals (Lhuguenot & Maume 1974); 3-methoxy-4-hydroxymandelic acid, 3-methoxy-4-hydroxyphenylacetic acid and 3-methoxy-4-hydroxyphenylglycol in amniotic fluid (Zambotti *et al.* 1975). While a report on adrenaline levels in plasma has appeared (Wang *et al.* 1975a) mass spectrometry has not been widely applied to plasma catecholamines despite the high sensitivity that appears possible.

Gas-liquid chromatography combined with mass spectrometry is undoubtedly the most specific and sensitive method yet devised for the measurement of catecholamines and their metabolites and is now the method of choice for some catecholamine metabolites previously difficult to determine by other techniques. It suffers from the disadvantages of being beyond the resources of most laboratories for routine catecholamine assay and requiring careful sample purification and separation.

3.10 RADIOENZYMATIC ASSAYS

The double-isotope derivative assay, where one radioisotope is used to label an internal standard and another to label the reagent, is a well established method in biochemistry and pharmacology. The availability of high specific activity chemicals and the ability to count low levels of radioactivity mean that extremely low concentrations can be determined.

The principle of the method is as follows. An aliquot of the material to be analysed is homogeneously mixed with a known amount of labelled compound (L_1). The labelled internal standard plus the unknown are separated from the bulk of the biological material and a derivative is prepared with a reagent labelled by a different radioisotope (L_2). For primary and secondary amines as well as alcohols labelled acetic anhydride is often used; any other suitably labelled reagent can be used provided that its specific activity is sufficiently high to achieve the sensitivity required of the assay. After the preparation of the derivative an appropriate purification procedure (such as solvent extraction or thin layer chromatography) is applied to separate the required L_2 -labelled derivative from the labelled reagent and any labelled by-products of the reaction. From the L_1 and L_2 activities it is then possible to calculate the recovery and the original concentration of the unknown (Riess 1974).

In the case of the catecholamines the principal approaches fall into 3 general classes of which 2 employ enzymes to effect the labelling.

1. The double isotope derivative assay of Engelman and his colleagues (Engelman *et al.* 1968, Engelman & Portnoy 1970) is based on the conversion of noradrenaline and adrenaline to their ^{14}C -methylated analogues by incubation with ^{14}C -S-adenosyl-methionine and catechol-O-methyltransferase in the presence of tracer quantities of ^3H -noradrenaline. The labelled normetanephrine and metanephrine so formed are isolated on an Amberlite IRC-50 column, separated on thin layer plates, oxidized to vanillin by sodium periodate and the radioactivity determined by liquid scintillation counting. Compared to the fluorimetric assay the important advantages offered by this assay are its specificity, greater sensitivity and the provision of an internal standard of recovery for every sample. The sensitivity of the method is such that samples containing as little as 0.25 ng of noradrenaline or adrenaline could be measured accurately with a sample count twice that of the assay blank. The method may be applied to all catechols O-methylated by catechol-O-methyltransferase and assays have been published for 3,4-dihydroxymandelic acid (Sato & DeQuattro 1969) and dopamine (Christensen 1973, Cuello *et al.* 1973), based on this procedure. A disadvantage is that careful isolation and separation techniques are necessary, involving many time-consuming steps. Also, precision and accuracy were not great in the early methods using radioenzymatic techniques.

Passon & Peuler (1973) modified the Engelman method and achieved a 10-fold increase in sensitivity and a considerable reduction in the operational time required. Their method is based on the enzymatic conversion of noradrenaline and adrenaline to their methylated derivatives in the presence of catechol-O-methyltransferase using ^3H -S-adenosylmethionine as the tritiated methyl donor. The column plasma purification steps of the Engelman method were eliminated. The much higher specific activity of ^3H -S-adenosylmethionine compared to the ^{14}C -labelled compound

resulted in greatly increased sensitivity, though at the expense of omitting the internal standard (since ^{14}C -catecholamines do not have the necessary specific activity). Only 0.75 ml of plasma is required and the method has been successfully applied to the measurement of noradrenaline and adrenaline in human plasma (Cryer *et al.* 1974, Moerman *et al.* 1976).

The assay has been extended to include dopamine as well as noradrenaline and adrenaline by Ben-Jonathan & Porter (1976) using a thin-layer chromatography system (2 dimensional) with only 1-2% overlap between 3-methoxytyramine, normetanephrine and metanephrine. An inter-assay coefficient of variation of 17.5% for dopamine, 26.9% for noradrenaline and 29.4% for adrenaline was reported. Sensitivity was 10-30 pg of each compound requiring only 25-100 μl of plasma. Each sample was corrected for recovery by the addition of ^{14}C -labelled, 3-O-methylated compounds after the incubation. Inhibition of catechol-O-methyltransferase activity by plasma constituents (possibly Ca^{2+}) could be largely overcome by the use of higher concentrations of Mg^{2+} and the addition of the complexing agent ethyleneglycol-bis(β -aminoethylether)N,N'-tetraacetic acid (EGTA) to the incubation mixture.

A more sensitive radioenzymatic assay reported to date is that of da Prada & Zürcher (1976), capable of determining all three catecholamines in plasma, to 5 pg of dopamine and 1 pg of noradrenaline or adrenaline. Principle improvements are the use of tetraphenylborate to complex the 3-O-methylated products of the reaction, reducing their polarity and markedly increasing their recovery into organic solvent and the thin layer chromatographic system where complete separation in 25 minutes is possible. With this procedure 40-50 samples can be handled by one person in 2 days.

The last 2 years have seen even greater advances in the technicalities of the radioenzymatic assay (Peuler & Johnson 1977, Fekete *et al.* 1978, Saller & Zigmond 1978). These improvements have been in the chromatographic systems employed, the range of catecholamine metabolites assayed, the greater specific activity

of the ^3H -S-adenosylmethionine available, the smaller assay volumes and the reduction in the number of steps involved. The maximum sensitivity attainable has culminated in the report of Saller & Zigmond (1978) summarized in Table 3-4.

Table 3-4
Radioenzymatic Assay Sensitivity

Standard (1 ng)	Count Minute ⁻¹ (n=4)	Blank (n=4)	Sensitivity* (pg)
Dopamine	230, 238±516	571± 78	5.0
Noradrenaline	203, 208±370	80± 28	0.8
Adrenaline	204, 147±402	72± 36	0.7
DOPAC	222, 212±320	802±188	7.2

* the amount of substance necessary to yield twice blank
[from Saller & Zigmond 1978]

2. Noradrenaline may be assayed by forming labelled adrenaline with phenylethanolamine N-methyltransferase (Saelens *et al.* 1967, Iversen & Jarrott 1970, Henry *et al.* 1975b, Weise & Kopin 1976). Henry *et al.* (1975b) found that the assay could determine accurately as little as 25 pg of noradrenaline with no interference from either dopamine or adrenaline. Again this assay is a single-isotope derivative procedure using tritiated S-adenosylmethionine as the methyl donor. The method is restricted to suitable primary amine substrates of phenylethanolamine N-methyltransferase; hence adrenaline cannot, while octopamine (Saavedra 1974) may, be determined. Weise & Kopin (1976) use catechol-O-methyltransferase to determine dopamine and the sum of noradrenaline and adrenaline concentrations and phenylethanolamine N-methyltransferase for noradrenaline alone. Hence adrenaline is calculated by difference. However compared to other assays more work is required and, in the presence of large quantities of noradrenaline, the accuracy of the adrenaline assay becomes uncertain.

3. A non-enzymatic approach is that taken by Franklin & Mayer (1968) and Rentzhog (1972) who acetylated the catecholamines with ^3H -acetic anhydride. Rentzhog (1972) reported that the catecholamine tri- and tetraacetates were stable enough for extensive purification and separation. The sensitivity was not great however, lying between 1 and 10 ng, depending on the tracer used. As with previous techniques the main disadvantage is the extensive chromatographic procedure necessary. The relatively high cost of these isotopic assays could also be important in some applications.

3.11 BINDING AND IMMUNOLOGICAL ASSAYS

Radioimmunoassay, protein-binding assay, radioreceptor assay and other saturation assay techniques have come into widespread use in the years since 1960 (Yalow & Berson 1960) in the measurement of substances that often cannot be satisfactorily measured by any other means (Ekins 1974).

Antibodies to most enzymes involved in catecholamine synthesis (§2.2) and metabolism (§2.4) have now been raised. For example, Goldstein *et al.* (1972) produced antisera to dopa decarboxylase, dopamine β -hydroxylase and phenylethanolamine N-methyltransferase and used them to investigate the tissue localisation and heterogeneity of the enzymes between different species. The use of assays for plasma dopamine β -hydroxylase has already been mentioned (§2.5), but recently several radioimmunoassays have appeared. An assay utilizing antibodies to sheep adrenal dopamine β -hydroxylase (Rush & Geffen 1972) has been modified to use human (Ebstein *et al.* 1973) and bovine β -hydroxylase (Kashimoto *et al.* 1974).

In addition antibodies have been prepared to dopamine and nor-adrenaline as well as to some related compounds. The catecholamines are themselves immunologically unreactive and must be coupled to a larger reactive molecule. A variety of methods are available for coupling to such haptens (Marks *et al.* 1974).

Aromatic amines may be diazotized and then coupled to proteins, or water-soluble carbodiimides may be used to couple amine groups with the carboxyl groups on the protein to form an amide linkage. Alternatively, the phenolic hydroxyl group can be linked to the protein. A noradrenaline-protein conjugate is possible via the carbodiimide reaction and antibodies formed by immunizing rabbits (Hartman & Spector 1972). A polypeptide containing dopamine covalently bonded to the γ -carboxyl group of glutamic acid has given rise to an antibody capable of inhibiting the stimulatory effects of catecholamines on lipolysis of isolated adipocytes (Spector *et al.* 1973). Since dopamine, noradrenaline and adrenaline are all active with respect to the antibody the assay is not specific. Peskar *et al.* (1972) produced a rabbit antibody to normetanephrine by coupling the hapten amino group with a synthetic copolymer using glutaraldehyde. The antibody recognized not only the ring structure but was also sensitive to changes in the side chain of the molecule. Normetanephrine and metanephrine elicited the same antigenic reaction while 3-methoxytyramine also showed a reaction though somewhat smaller. Dopamine, noradrenaline and adrenaline had no effect. Ten ng of normetanephrine and/or metanephrine could be detected; too high for assay of cerebrospinal fluid samples without concentration.

Rather than employ catecholamines for hapten-carrier conjugation by formaldehyde conjugation Grota & Brown (1976) employed the phenolic analogues of dopamine, noradrenaline and adrenaline (p -tyramine, octopamine and synephrine respectively) for two reasons

- (1) they possess much greater chemical stability
- (2) they are close in structure to the amines of interest

From previous data it was anticipated that the resulting antibodies would cross-react sufficiently with the corresponding catecholamines to allow their assay. For example the antisera raised against p -tyramine exhibited decreased binding of compounds with N-methyl groups or β -hydroxyl groups (such as noradrenaline and adrenaline) or compounds with no p -hydroxyl group,

while hydroxyl substitution at ring position 3 (ie. dopamine showed significant binding (though less than by ρ -tyramine) and methoxy substitution (ie. 3-methyltyramine) gave the most reactive antigen of all. For antibodies raised against synephrine an ultimate sensitivity of 100-1000 pg of adrenaline and 25-100 pg of metanephrine was indicated, giving this system the potential of a practical assay. Four hundred pg of 3-methoxytyramine can be detected with an antibody developed by Faraj & Camp (1976), combining sensitivity with a high degree of selectivity.

If natural tissue receptors for the catecholamines (Seeman *et al.* 1975, Myers *et al.* 1976) could be sufficiently purified there is also the possibility of developing a binding assay of sufficient sensitivity and selectivity for low concentrations of catecholamines. The catecholamine-binding properties of serum proteins (§3.2) have been utilized in an assay with a sensitivity of less than 2 ng; but the reported results with normal human plasma are almost 100 times greater than the currently accepted range (§5.2) (Araki *et al.* 1976).

3.12 CONCLUSIONS

The information presented in previous sections is summarized below with an estimate of the ultimate sensitivity of each method.

Bioassay (0.5 ng ml⁻¹) Relatively specific for catecholamines but difficult to distinguish between noradrenaline and adrenaline. Subject to interference and not easy to set up and standardize. Inexpensive and relatively sensitive, but lacks accuracy and precision.

Colorimetric (1.0 μ g ml⁻¹) Insensitive and does not distinguish between different catecholamines.

Fluorimetric (0.1 ng ml⁻¹) Relatively specific for catecholamines and acceptable resolution between noradrenaline and adrenaline may be achieved.

Successful plasma assays require considerable attention to detail. A wide range of catecholamine metabolites can be fluorimetrically estimated.

TLC (5 ng) Highly specific but quantitative estimation is difficult. Relatively insensitive.

Liquid Chromatographic (0.2 ng ml⁻¹) Good resolution and with electrochemical detection, good sensitivity.

GLC (0.1 ng ml⁻¹) Highly specific and excellent resolution but requires initial chemical transformation and most procedures lack somewhat in sensitivity.

GLC-Mass Spectrograph (0.001 ng ml⁻¹) Superb resolution and sensitivity but expensive and not yet generally applied to plasma catecholamines. Impractical for many laboratories.

Radioenzymatic (0.01 ng ml⁻¹) Excellent sensitivity but poor resolving power which means time-consuming separation procedures. Relatively expensive in terms of chemicals. The high sensitivity permits small blood volumes to be used.

Binding (0.2 ng ml⁻¹) Not yet developed to the stage of a practical assay. Specificity is still a problem but the method would appear to have potential.

Improvements in the fluorimetric method over the last decade and the introduction of isotopic methods have enabled measurements of plasma catecholamines to be made with a degree of confidence despite the inherent difficulties in these techniques. However it must be concluded that a simple assay for plasma catecholamines requiring a small blood volume is still not available.

METHODS OF ASSAY

CHAPTER 4

4.1 INTRODUCTION

In this chapter the assay procedures used in the analysis of over 2000 samples discussed in subsequent chapters are covered. Each blood, cerebrospinal fluid or tissue sample collected during the course of this work was uniquely numbered and details of the sampling circumstances recorded in an experiment book.

Reagents The chemicals used were of laboratory grade or wherever possible of analytical grade. All chemicals and biochemicals mentioned in this or other chapters are listed alphabetically in Appendix II together with the chemical grade, the manufacturers name, the chemical formula and the molecular weight.

Water The fluorimetric assay of catecholamines (§4.4) requires highly purified water. All solutions for analytical or animal injection purposes were prepared using double distilled water from an all-glass, electrical double still (Jobling Laboratory Division, Stone, Staffordshire, England). Disodium ethylenediamine tetraacetic acid (approximately 1 g) was added to the first boiler to prevent scale build-up and to improve final water quality.

Glassware Glassware was cleaned by soaking in a dilute chromic acid solution (1175 g of potassium dichromate, 1 l of concentrated sulphuric acid to 10 l of distilled water) for at least 48 hours, removed, rinsed in distilled water, and soaked for 2 hours or more in a dilute solution of disodium ethylenediamine tetraacetic acid (approximately 0.2% w/v) and sodium sulphite (0.04% w/v) to remove and reduce traces of chromic and other heavy metal ions.

4.2 RELIABILITY OF ANALYSIS

Reliability is commonly described as an amalgam of the specificity, accuracy, precision and sensitivity attributes of the assay in question. In other words it is important to know how near an analytical result approaches the true result (accuracy), the reproducibility of the results (precision), the least amount of the substance analysed which is distinguishable from zero (sensitivity) and whether the analysis measures what it is supposed to and measures nothing else (specificity). Some basic information about the reliability of the analyses employed is desirable since conclusions regarding changes in the levels of biological substances must be weighed accordingly. The definitions of assay properties below are in common use and are described in more detail elsewhere (Anastassiadis & Common 1968). Of course the acceptability of an assay depends not only upon its reliability but also on its applicability which encompasses such factors as sample size, sample type, speed of analysis, equipment necessary, time available and the overall cost. Since there are many difficulties in the assay of plasma catecholamines considerations of assay methods are almost totally centred on the reliability aspects while, at least in the research laboratory, questions of applicability are secondary.

Specificity The specificity of a method is the degree to which the mean measurement obtained by the method is due to the substance to be determined and not to other substances that may be present in the matrix under analysis. Conclusive evidence for the absolute specificity of any method is difficult to attain however, since the sources of potential interference are usually considerable and all must be investigated. Save for the mutual interference between catecholamines, specificity has not been investigated to any degree in the assays described in this chapter. Numerous investigators have examined the contribution of other substances to catecholamine assays. These have been noted but it is considered unlikely that those substances shown to interfere in the fluorimetric catecholamine assay were present in the plasma of the unmedicated sheep used in the present experiments.

Accuracy The accuracy of an analytical procedure for the determination of a substance may be defined as the degree to which the mean measurement and therefore the mean estimate obtained by the method approaches the true measurement, and therefore the true value, for the substance after the effects of other substances giving the same measure have been eliminated either physically or mathematically. Inaccuracy in the procedure may be due to losses during the procedure or to alterations of the measured property of the substance caused by the procedure. The absolute amount of a substance can seldom be known with certainty. All that can be done is to provide evidence for or against the analytical result being near to the true result, such as by the use of internal standards.

Precision The precision of an analytical method may be defined as the degree to which a "representative determination" of the substance will yield a measurement that approaches the average measurement of an infinite number of determinations on the same material. The term precision therefore denotes the reproducibility of the analytical results and should not be confused with accuracy or sensitivity.

Sensitivity The sensitivity of a method may be defined as the ratio of the displacement of an element of the instrument used in the measurement of the property of the unknown substance to the amount present. Sensitivity may be measured and expressed as the smallest difference between the contents of the unknown substance in 2 different samples that can be distinguished by the method.

4.3 AN APPARATUS FOR THE RAPID EXTRACTION AND CONCENTRATION OF CATECHOLAMINES FROM BIOLOGICAL FLUIDS

INTRODUCTION

For the analysis of catecholamines in biological fluids many methods including fluorimetry (§3.6) and gas-liquid chromatography (§3.9) first require initial sample purification. At the same time some concentration of the low levels of catecholamines in plasma is also desirable. Various isolation methods have been described based on aluminium oxide or ion-exchange chromatography or both (§3.3). All these methods involve many steps and are time consuming.

To meet a need to handle plasma extractions more rapidly than is possible with published procedures an extractor has been developed based on the batch aluminium oxide adsorption procedure of Anton & Sayre (1962). The principle of the method is to perform all the necessary adsorption, washing and elution steps in the same vessel above a fritted glass disc of appropriate porosity through which nitrogen may be passed upwards (suspending the aluminium oxide particles in solution) or suction applied from below to withdraw the liquid contents of the extractor.

Preparation of the Aluminium Oxide

Aluminium oxide was acid washed and activated essentially by the method of Anton & Sayre (1962) excepting that the final activation was carried out by heating at 300° for 3 hours.

- (a) 100 g of aluminium oxide was added to 500 ml of 2N hydrochloric acid in a covered 2 l beaker and heated for 45 minutes at 90° with continuous and rapid stirring on a combination hot plate and magnetic stirrer (Cenco Instrumenten MJBV, The Netherlands) with a 60 mm magnetic follower.

- (b) The beaker was removed from the stirrer and the heavier particles of aluminium oxide allowed to settle for $1\frac{1}{2}$ minutes. The supernatant fluid (yellow in colour) was decanted along with the finer particles of aluminium oxide.
- (c) The aluminium oxide was washed twice with fresh 250 ml portions of 2N hydrochloric acid at 70° for 10 minutes discarding the supernatant with the finer aluminium oxide particles each time.
- (d) In the final acid wash, the aluminium oxide was stirred with 500 ml of 2N hydrochloric acid at 50° for 10 minutes.
- (e) After decanting the hydrochloric acid the aluminium oxide was washed repeatedly (20-40 times) with fresh 200 ml portions of distilled water until a wash-water pH of 3.4 was reached, decanting the finer particles each time.
- (f) Finally the aluminium oxide was transferred to an evaporating dish, heated at 105° for 1 hour and at 300° for 3 hours in an electric furnace (A. Gallenkamp & Co, London, UK). On cooling in a dry atmosphere the activated aluminium oxide was split into 10 g lots in capped vials and stored in an incubator (Andrew Thom Ltd, Sydney, Australia) at 37° to keep the powder dry. In this manner 80 g of aluminium oxide was prepared, sufficient to extract over 400 plasma samples.

Extractor

A 10 mm diameter filter tube (Number FD-532, A. Gallenkamp & Co, London, UK), containing at its midpoint a fritted glass disc of porosity 2 (nominal pore diameter 40-50 μm), was shortened at one end to 20 mm, then extended by 60 mm of glass tubing (2 mm id) (Figure 4-1). Tubing of 30 mm diameter and 100 mm in length was fused to the opposite end of the filter tube to form the

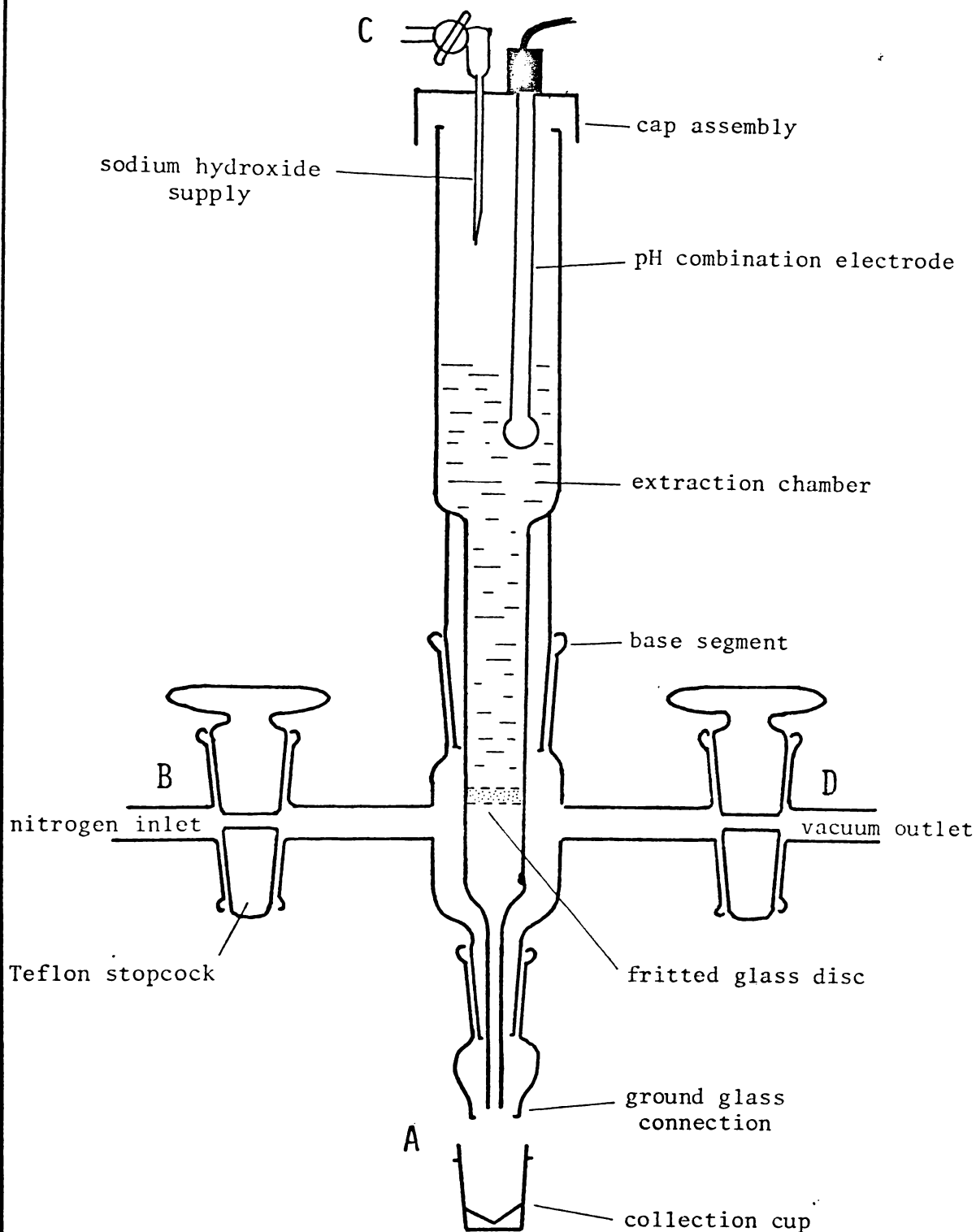


Figure 4-1. Apparatus for the extraction of catecholamines from biological fluids.

extraction chamber. At the same junction was fused a cone (joint size 24/29) forming a skirt about the filter tube and extending down to within 10 mm of the fritted glass disc. In the assembled apparatus the cone was seated in the socket joint of the base segment which contained an inlet for nitrogen and a suction outlet controlled by Teflon stopcocks. The base was fixed to laboratory scaffolding and a light metal spring attached between the extractor and the base to prevent nitrogen escape under pressure. With an eluate volume greater than 1.5 ml a test tube with a socket connection 14/23 may be attached to the base section for eluate collection. For the extraction of catecholamines in a small eluate volume, as described below, a glass extension was added to which a plastic cup (2 ml capacity, Technicon, Tarrytown, New York, USA) was connected. The glass adaptor was ground at the connection (12 mm od) to prevent cup separation under nitrogen pressure. Plastic tubing from a water pump (12 mm id) may also be attached at this point to draw off to waste the extracted biological sample and the wash fluids.

A combination pH electrode (Number S-30070-10, Sargent-Welch Scientific Co, Skokie, Illinois) and an EIL 7050 pH meter was used to adjust the sample pH. Two small holes were made in a plastic cup fitting loosely over the top of the extractor through which a needle for the gravity-fed sodium hydroxide (0.2 N) supply and the electrode were inserted. After the pH of a sample was adjusted and the electrode rinsed in distilled water the cap assembly was moved to the next extractor.

Suction was generated by 2 water pumps one of which was attached to the base segment of each extractor and the other to 2 waste pipes each having a Teflon stopcock (Figure 4-2). The nitrogen was supplied from a cylinder. Completion of the adsorption and elution steps for each sample was indicated by a multi-channel electronic timer based on the mains frequency. At the beginning of the adsorption or the elution phase of each extraction a corresponding numbered toggle switch on the timer was set by moving it into the upwards (adsorption) or the downwards (elution)

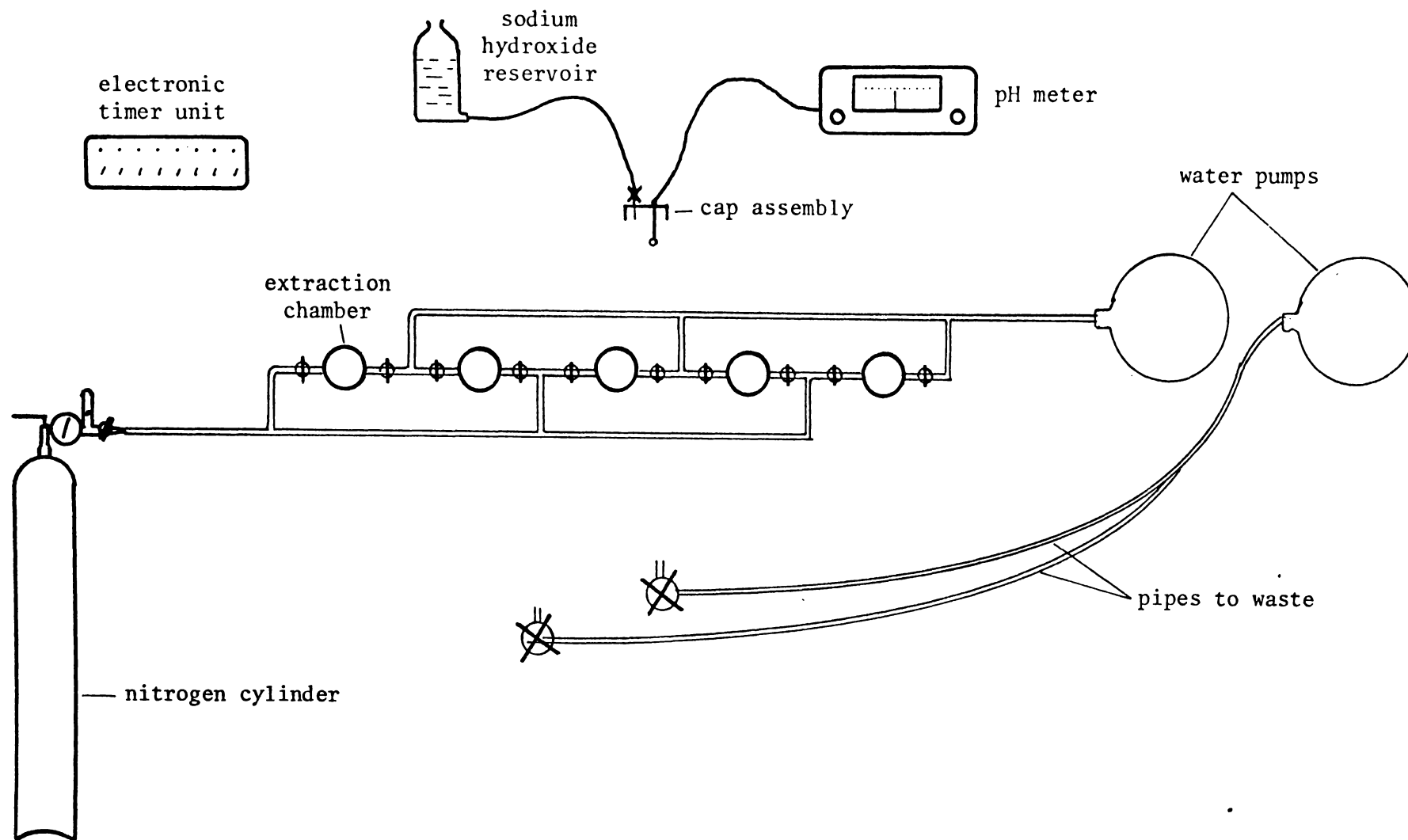


Figure 4-2. Block diagram of a 5-unit catecholamine extraction apparatus.

position. Completion of the time interval was signalled by a light and an auditory alarm which were terminated by centering the switch.

Extraction Procedure

The protocol for the extraction of catecholamines from biological fluids was based on a detailed investigation of the factors influencing catecholamine recovery discussed below.

1. Approximately 175 mg of aluminium oxide was added to the extractor with a glass measure and a 2 ml plastic cup attached at A (Figure 4-1).
2. The nitrogen was turned on at B and 4 ml of 0.3 N sodium hydroxide solution containing sodium sulphite (0.25% w/v), disodium ethylenediamine tetraacetic acid (2.5% w/v) and Dow Corning Antifoam B (0.5% v/v) added to the extraction chamber.
3. The deproteinized biological sample (§4.4) was added and the pH adjusted to 8.5 with 0.2 N sodium hydroxide via a stopcock at C and the timer activated.
4. After completion of the adsorption step (5 minutes) the nitrogen was turned off, the plastic cup replaced by the waste pipe at A and the extracted plasma solution removed. The aluminium oxide was washed with several portions of double distilled water from a wash bottle, totalling at least 50 ml.
5. A clear plastic cup was attached at A, the nitrogen again turned on at B and 1 ml of 0.1 N hydrochloric acid added to the aluminium oxide with a piston pipette (Eppendorf Gerätebau Netheler & Hinz GmbH, Hamburg, West Germany). The corresponding timer switch was activated. At the completion of the elution (6 minutes), the nitrogen was turned off and suction applied via the stopcock at D, drawing the eluate through the fritted glass disc into the collection cup. The sample was then removed for analysis or capped and stored at -20° .

6. The extractor vessel was separated from the base and the aluminium oxide washed out with tap water. After replacement in the base segment the extractor was briefly washed with distilled water drawn through the fritted glass disc - ready for the next extraction.

EXPERIMENTAL METHODS

Taking the results of Anton & Sayre (1962) into account and after a brief preliminary survey of the approximate optimal conditions for efficient extraction, a detailed investigation of the recoveries of l-dopa, dopamine, noradrenaline and adrenaline was carried out. One variable at a time was altered in each of a series of 6 experiments, holding all other conditions to those described above (except that an eluting acid strength of 0.25 N was used). Standard 4 ml solutions of each separate catecholamine (5 ng ml^{-1}) were extracted in triplicate to determine each experimental point. Approximately 40 000 dpm (disintegrations per minute) of the corresponding tritiated analogue (repurified before use by aluminium oxide adsorption) were also added to the standard solution. 0.5 ml of the final eluate was mixed with 10 ml of Biofluor emulsifier cocktail (New England Nuclear, Boston, Massachusetts, USA) and counted on a Packard Tricarb 2425 liquid scintillation counter for the calculation of recoveries. Recoveries are expressed as % per millilitre of eluate rather than as absolute recoveries (which depend on the fraction of the eluate volume finally collected). In the fluorimetric assay of catecholamines the concentration is more important than the total recovery provided that sufficient eluate is available for the microfluorimetric determination of noradrenaline and adrenaline in duplicate together with blanks. If a high absolute recovery is required a small volume of water or dilute acid may be used to wash the remaining eluate from the fritted glass and the aluminium oxide.

600 ml of pooled ovine blood was collected at an abattoir, the plasma separated and split into 4 sets of seventeen 4 ml samples. To 11 of each set were added 200 ng of either l-dopa, dopamine, noradrenaline or adrenaline together with 40 000 dpm of the corresponding tritiated catecholamine. Three aliquots of each set were used for the determination of the endogenous catecholamine levels - only the radiolabelled catecholamine was added. The samples were extracted as described and the recoveries calculated by counting 0.2 ml of eluate. With the noradrenaline and adrenaline samples 0.5 ml of the remaining eluate was diluted to 2 ml with 0.1 N hydrochloric acid and analysed using the fluorimetric catecholamine assay (§4.4). The deproteinized supernatants of a further 12 plasma samples, 3 for each catecholamine, were added to the extraction chamber and the exogenous and tritiated catecholamine added separately to determine the effect of deproteinization on recoveries.

RESULTS

Dopamine, noradrenaline and adrenaline had similar adsorption characteristics over the range of conditions investigated (Figure 4-3). L-dopa under most circumstances also behaved similarly but its recovery fell more rapidly with short adsorption times, low elution acid strengths and also when the total quantity of l-dopa in the extracted solution was increased to 4 µg.

Quantity of Aluminium Oxide Recoveries dropped markedly when less than 100 mg of aluminium oxide were used for adsorption (Figure 4-3a). The optimum weight is 150-200 mg; significantly less than other commonly used batch procedures [300 mg (Griffiths *et al.* 1970), 400 mg (Anton & Sayre 1962), 500 mg (Renzini *et al.* 1970) or the 2 g in the column procedure of Valori *et al.* (1969)]. The optimum amount of aluminium oxide will be determined to some extent by the extracted volume and the particle size, though these aspects have not been explored.

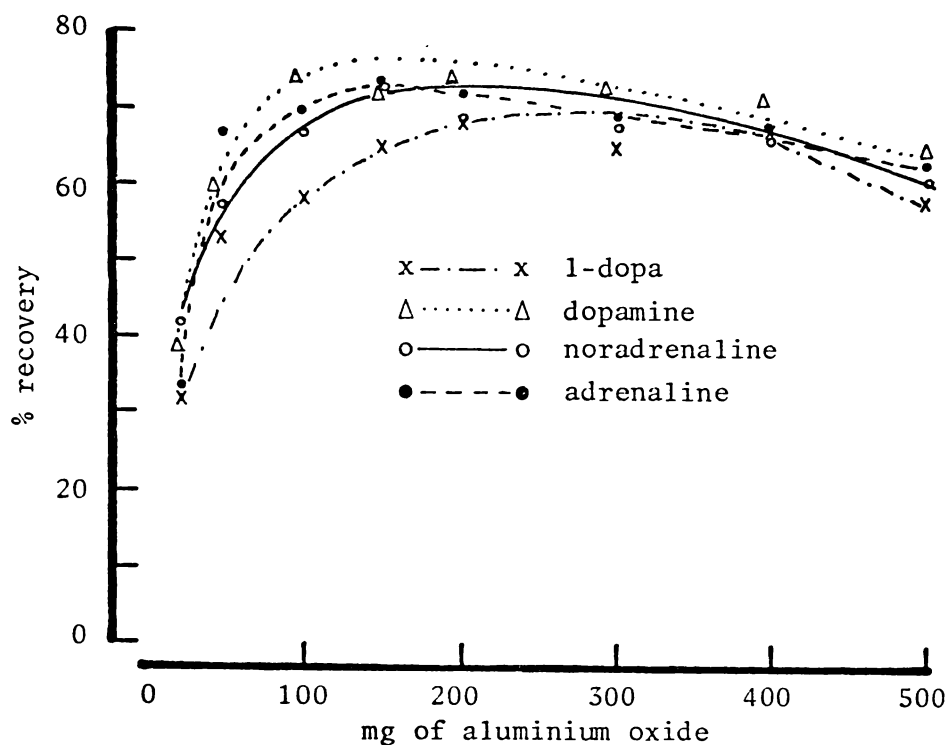


Figure 4-3a. Catecholamine recoveries from standard solutions vs the quantity of aluminium oxide used for adsorption.

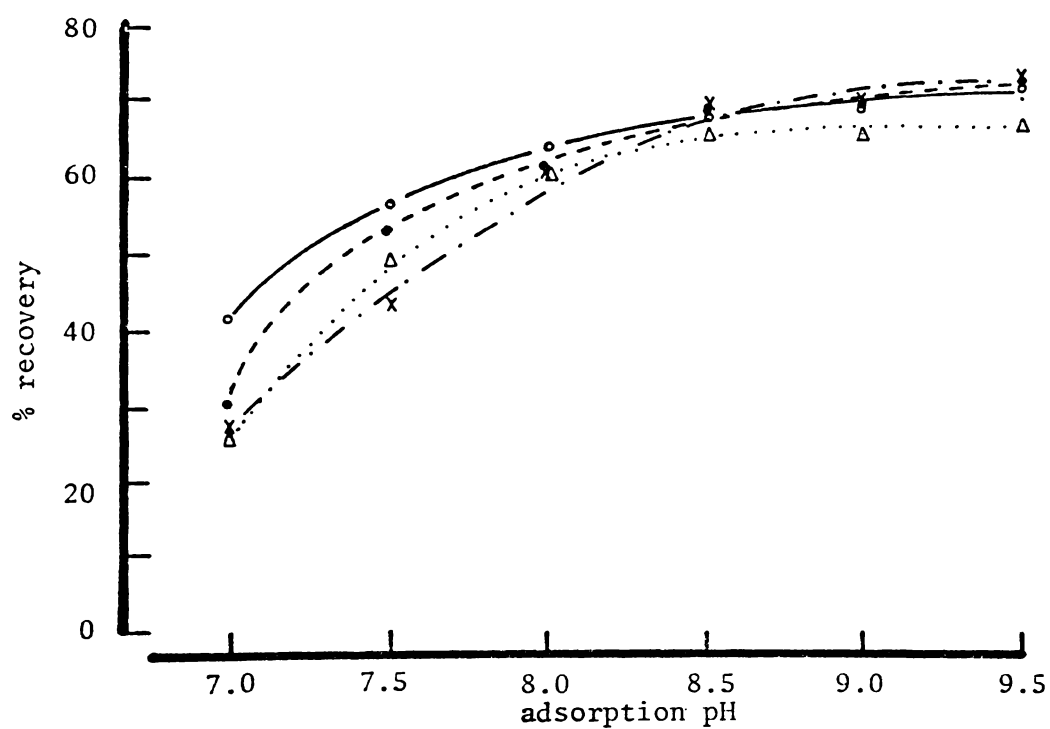


Figure 4-3b. Catecholamine recoveries from standard solutions vs the adsorption pH.

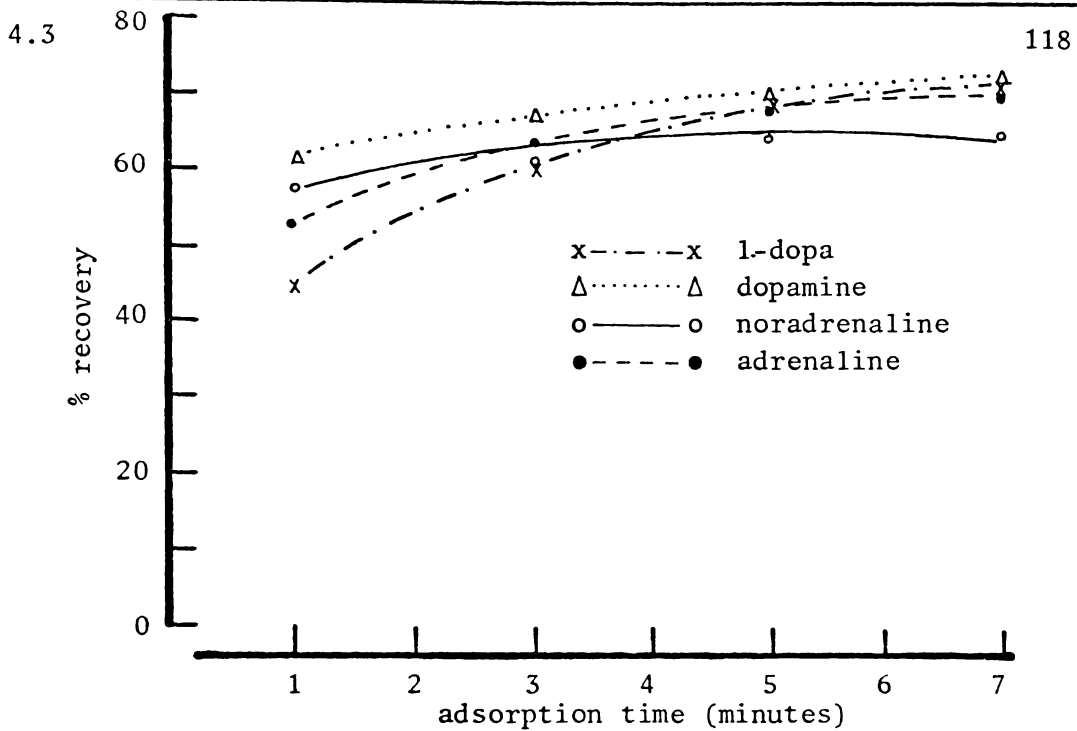


Figure 4-3c. Catecholamine recoveries from standard solutions vs the adsorption time.

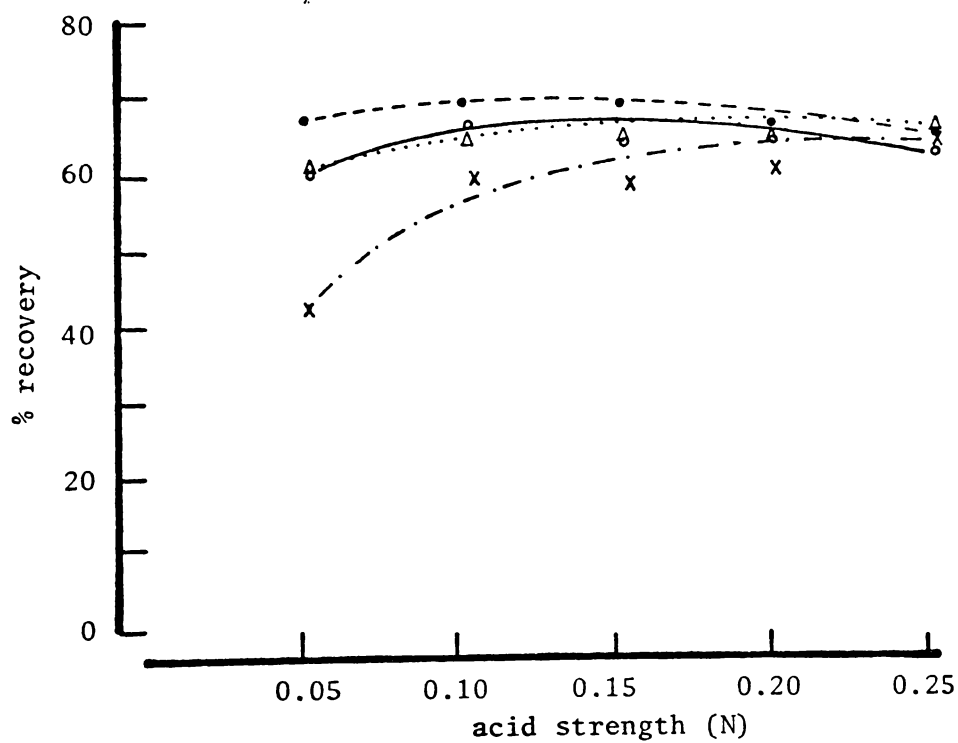


Figure 4-3d. Catecholamine recoveries from standard solutions vs the eluting acid strength.

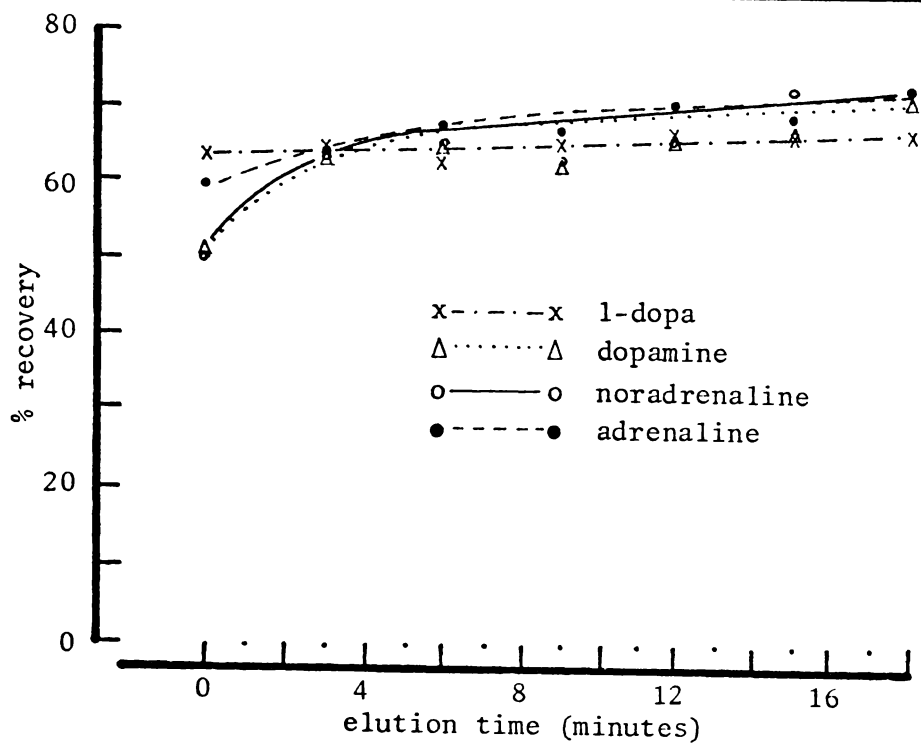


Figure 4-3e. Catecholamine recoveries from standard solutions vs the elution time.

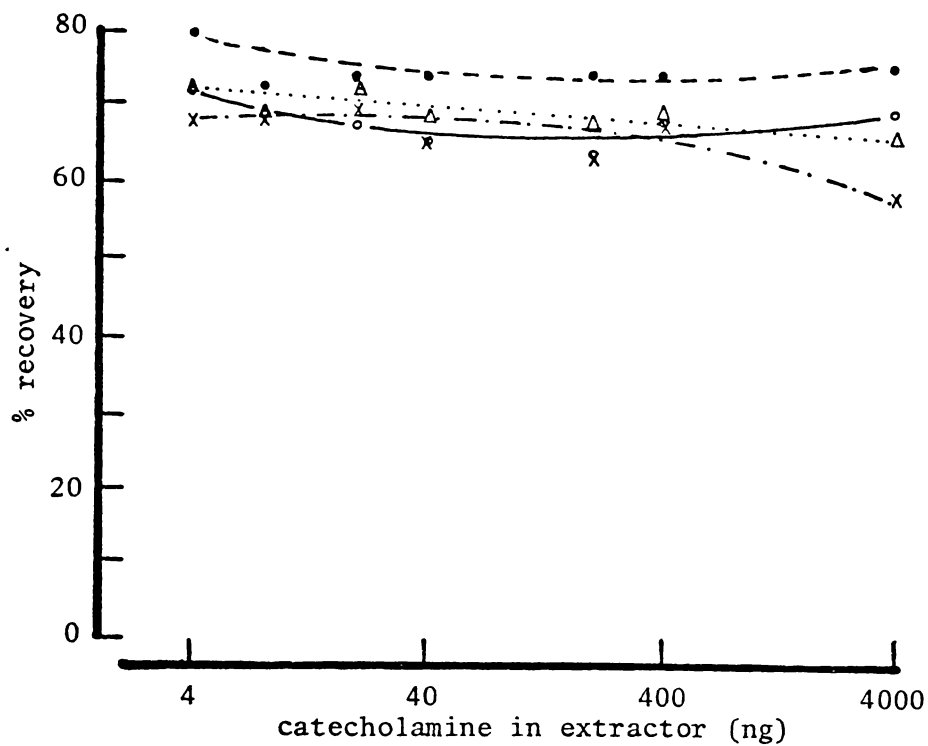


Figure 4-3f. Catecholamine recoveries from standard solutions vs the catecholamine load.

Adsorption pH A marked fall in the recovery of catecholamines, comparable to that found by Anton & Sayre (1962) (Figure 3-1), was found when the adsorption pH fell below 8.0 (Figure 4-3b). In contrast to Anton & Sayre (1962) recoveries continued to increase slowly with an increasingly alkaline solution to pH 9.5. The tendency of catecholamines to oxidize in alkaline media may be prevented in the present procedure by the nitrogen atmosphere.

Adsorption Time An increase in recovery occurred with increasing time of adsorption up to 7 minutes (Figure 4-3c). L-dopa was especially adversely affected by a short adsorption time with recoveries of 44.7% to 73.5% through this range.

Elution Acid Strength Below an elution acid normality of 0.1 recoveries dropped; above this level little further improvement was found (Figure 4-3d). While a standard acid strength of 0.25 N was used in this experiment, the necessity for precise pH adjustments in the differential fluorimetric catecholamine assay (Lavery & Taylor 1968) suggests an acid strength of 0.1 N is appropriate for the analysis of biological fluids.

Elution Time The range of elution times from 0 (acid drawn directly through the aluminium oxide) to 18 minutes had less effect on recoveries than expected, rising on average for all catecholamines from 57.0 to 71.7% (Figure 4-3e). L-dopa was especially readily eluted.

Catecholamine Load Recoveries varied little over a 1000-fold increase in the amount of catecholamine added to the extraction chamber (Figure 4-3f). Only L-dopa showed any indication of a fall in recovery at the highest load (4 μ g). In most biological fluids therefore recoveries would not be expected to depend on the catecholamine content.

The antifoam used to prevent foaming of plasma samples had no effect on recoveries, nor were significant statistical differences found between the efficiencies of 4 individual extractor vessels (analysis of variance $F_{3,44} = 0.806$, $P > 0.2$). Cross contamination between samples extracted in the same vessel was less than 1% (Table 4-1) and on average 0.874 ml of eluate was recovered (1 ml eluant volume).

Table 4-1

Average ($\pm$ SD) Values for Some Extraction Parameters (n=11)

Weight of aluminium oxide dispensed by scoop	175.5 $\pm$ 6.3	mg
Eluate volume (1 ml eluant)	0.874 $\pm$ 0.041	ml
Eluate normality (0.25N HCl eluant)	0.184 $\pm$ 0.006	N
Cross contamination between samples	0.75 $\pm$ 0.63	%

While significant differences between the 4 catecholamines did not emerge when extractions were carried out from standard solutions, plasma extractions showed significantly higher nor-adrenaline and adrenaline recoveries than either dopamine or 1-dopa (Table 4-2) (analysis of variance between catecholamines $F_{3,40} = 5.28$, $p < 0.01$). Protein precipitation reduced the average recovery of catecholamines by about 20%. This loss occurred in the protein precipitation rather than during extraction since radiochemical recoveries were similar to those from standard solutions when the deproteinized plasma and the radio-label were added separately to the extractor.

The recovery of adrenaline assessed fluorimetrically (51.9 $\pm$ 4.1) did not differ significantly from the value obtained radiochemically (Table 4-2). On the other hand a small but significant ($p < 0.001$, t-test) difference was evident between the mean nor-adrenaline recovery determined fluorimetrically (49.2 $\pm$ 3.9) and

Table 4-2

% Recovery (per ml of eluate) ($\pm$ SD) of Catecholamines
from Standard Solutions and from Plasma

	L-dopa	Dopamine	Nor- adrenaline	Adrenaline	Mean $\pm$ SD
Standard Solutions (n=18)	67.7 $\pm$ 4.3	69.8 $\pm$ 5.2	66.9 $\pm$ 2.8	64.2 $\pm$ 6.7	67.2 $\pm$ 5.2
Plasma* (n=11)	42.5 $\pm$ 2.3	42.2 $\pm$ 4.7	54.9 $\pm$ 2.5	52.1 $\pm$ 1.6	47.9 $\pm$ 6.4
Plasma** (n=3)	65.9	71.5	65.7	74.1	69.4

* label and exogenous catecholamine added to the plasma before protein precipitation

** label and exogenous catecholamine added to extraction chamber separately from the deproteinized plasma

the mean radiochemical determination (54.9 $\pm$ 2.5). The reason for this discrepancy is unknown.

CONCLUSIONS

While no significant differences were evident between the recoveries of l-dopa, dopamine, noradrenaline and adrenaline from standard solutions under the standard extraction conditions, l-dopa has a lower affinity for aluminium oxide and was less readily adsorbed and more readily eluted than the other catecholamines at the extremes of the investigated variables.

Both l-dopa and dopamine recoveries were depressed in the presence of plasma constituents.

With the extraction apparatus described up to 75 samples per day (compared with 25 or less by existing methods) may be processed with simultaneous improvements in the eluate concentration and recovery variability. Some groups (Anton & Sayre 1962, O'Hanlon *et al.* 1970, Campuzano *et al.* 1975) report recoveries of catecholamines as high as 90-100% but these results do not include losses due to protein precipitation in plasma. In the case of Anton & Sayre (1962) the eluant volume was 3 ml while the recoveries of O'Hanlon *et al.* (1970) and Campuzano *et al.* (1975) were calculated on the basis of 1.5 ml of eluate, 0.3 ml of which was trapped in the aluminium oxide particles.

Losses in protein precipitation have not been taken adequately into account in most fluorimetric assays. In this laboratory tritiated label is added to the plasma as soon as it is separated from whole blood. The proportion of the catecholamine in the original plasma sample used in the assay is then easily calculated and correction can be made for losses (Appendix III).

4.4 THE FLUORIMETRIC ANALYSIS OF CATECHOLAMINES

The background of developments in the fluorimetric assay of catecholamines is reviewed in §3.6. The assay evolved in this laboratory differs in several methodological respects from previously published procedures.

1. The assay sample and reagent volumes have been scaled down so that only 100 μ l of extracted plasma eluate is used.
2. The sample to reagent volume ratio is increased.
3. All samples are individually corrected for recovery using a tritiated catecholamine.
4. All reagents, standards and samples are filtered to remove fine particulate matter.

The basic chemical procedure is that of Laverty & Taylor (1968) with minor deviations. A number of other methods has also been investigated (Renzini *et al.* 1970, Diamant & Byers 1975) but these did not give the fluorophore stability combined with the sensitivity of the Laverty & Taylor method. The extraction procedure (§4.3) and the data analysis (Appendix III) which differ from other methods are described separately.

Blood Collection

The importance of rapid plasma separation from whole blood and the need for preservation to protect catecholamines against oxidation, cellular uptake and enzymatic attack have already been emphasized (§3.2). Blood samples were collected into 10 ml plastic disposable test tubes (15 x 100 mm) containing approximately 10 mg of sodium sulphite and 30 mg of disodium ethylenediamine tetraacetic acid. These salts were dispensed with a scoop from a vessel containing a mixture of 1 part of sodium sulphite to 3 parts of disodium ethylenediamine tetraacetic acid (w/w). Fifty μl of a 5000 units ml^{-1} preparation of sodium heparin were also added to the collection tube shortly before blood sampling to prevent clotting. Once blood collection was completed the tube was gently inverted several times and placed in a refrigerated water bath (4°) until centrifuging (2000 g at 4°). The plasma was separated by pipetting into a clean 15 x 100 mm test tube and the volume recorded. 0.5 ml of a purified tritiated dopamine, noradrenaline or adrenaline solution was added (~20 000 dpm) and thoroughly mixed with a glass rod. An equal volume of an ice-cold 0.8 N perchloric acid solution was then added to the plasma to precipitate the protein. The contents of the tube were again mixed thoroughly, the glass rod withdrawn and the adhering protein washed back into the tube with distilled water from a wash bottle. The precipitated plasma samples were stored at 4° and within 24 hours centrifuged at 1500 g for 10 minutes and the supernatant extracted according to the method described in §4.3.

Analytical Method

Equipment

Aminco-Bowman Spectrophotofluorometer with magnetic arc stabilizer, ratio photometer, off-axis ellipsoidal condensing mirror and xenon lamp (American Instrument Company, Silver Spring, Maryland, USA)

MSE Mistral 4L refrigerated centrifuge (Measuring and Scientific Equipment Ltd, Crawley, UK)

Sargent-Welch pen recorder, model DSRLG (Sargent-Welch Scientific Company, Skokie, Illinois, USA)

7 x 7 x 32 mm quartz cuvettes (minimum volume of 0.2 ml)
(American Instrument Company, Silver Spring, Maryland, USA)

12 x 75 mm polypropylene disposable test tubes with plastic stoppers (F.R. Galantai Manufacturing Company Ltd, Christchurch, New Zealand)

Eppendorf piston pipettes (10, 20, 50, 100 μ l) (Eppendorf Gerätebau Netheler & Hinz GmbH, Hamburg, West Germany)

Swinnex-13 10 mm diameter filter holder and MF-Millipore 10 mm plain white filters, pore size 0.45 μ m (Millipore Filter Corporation, Bedford, Massachusetts, USA)

Assay Solutions

All standard stock solutions were stored at 4° and were stable over at least 3 months. One μ g ml⁻¹ and further dilutions were prepared each day from these stock solutions. With the exception of the iodine all assay reagents were made up in bulk, split into aliquots, each sufficient to cover the requirements of the assay over half a day, and stored at -20°. As the reagents deteriorate in the aqueous phase they were not used after standing at room temperature for longer than 3 hours.

1-dopa ($100 \mu\text{g ml}^{-1}$) 10.0 of 1-dopa was dissolved in 100 ml of 0.001 N HCl

dopamine ($100 \mu\text{g ml}^{-1}$) 12.4 mg of dopamine hydrochloride was dissolved in 100 ml of 0.001 N HCl

noradrenaline ($100 \mu\text{g ml}^{-1}$) 19.9 mg of noradrenaline bitartrate monohydrate was dissolved in 100 ml of 0.001 N HCl

adrenaline ($100 \mu\text{g ml}^{-1}$) 10.0 mg of adrenaline was dissolved in 100 ml of 0.001 HCl

liquid scintillation cocktail

A convenient and low cost liquid scintillation cocktail for aqueous samples was prepared by adding 15 g of 2,5-diphenyl-oxazole (PPO) and 0.75 g of 1,4-bis-2-(5-phenyloxazolyl)-benzene (POPOP) to a winchester of toluene (2.5 l) and allowing it to stand for 24 hours for the additives to dissolve. When required the toluene solution was mixed with Triton X-100 in the ratio of 2:1 (v/v) (Price 1973).

citrate-phosphate buffers (pH 4.0 and 5.4)

10.51 g of citric acid was dissolved in 250 ml of distilled water (0.2 M) and 7.09 g of disodium hydrogen phosphate in 250 ml of distilled water (0.2 M). The phosphate solution was slowly added to the citrate solution until the desired pH is reached. Sufficient 2.5 N sodium hydroxide was then added so that 50 μl of the resulting solution exactly neutralized 100 μl of the 0.184 N hydrochloric acid eluant (§4.3). The buffer was split into forty 6 ml aliquots and frozen.

phosphate buffers (pH 6.5 and 7.0)

7.80 g of sodium dihydrogen phosphate was dissolved in 250 ml of distilled water (0.2 M) and 7.09 g of disodium hydrogen phosphate in 250 ml of distilled water (0.2 M). The disodium was added to the dihydrogen salt to give the desired pH, 2.5 N sodium hydroxide added as for the citrate buffer and the resulting solution split into forty 6 ml aliquots and frozen.

iodine (0.01 N)

2 ml of 0.01 N iodine was prepared twice daily from 0.2 N aqueous iodine stock solution.

alkaline sulphite solution

4.46 g of disodium ethylenediamine tetraacetic acid and 3.02 g of sodium sulphite were dissolved in 120 ml of 2.5 N sodium hydroxide and divided into 6 ml aliquots for freezing (noradrenaline assay).

2.23 g of disodium ethylenediaminetetraacetic acid and 1.51 g of sodium sulphite were dissolved in 120 ml of 1.25 N sodium hydroxide and divided into 6 ml aliquots for freezing (l-dopa, dopamine and adrenaline assays).

acetic acid solutions

Glacial acetic acid was diluted with distilled water in the following proportions to make the final assay reagent for each catecholamine.

Table 4-3

Glacial Acetic Acid Dilutions to Prepare Final Reagent Solutions

	L-dopa	Dopamine	Noradrenaline	Adrenaline
ml of water	110	100	30	51
ml of acetic acid	10	20	37.5	12

The proportions should be considered a guide only. The pH of the complete reaction mixture for a freshly prepared reagent set should be determined and if necessary appropriate adjustments made to the acetic acid solution to ensure optimal final acidity (Table 4-5). The bulk solutions were subdivided into conveniently sized aliquots (6 ml for l-dopa and dopamine assays and 2.5 ml for noradrenaline and adrenaline assays).

Analytical Procedure

Although each catecholamine was assayed according to the specific details in Tables 4-4 and 4-5 the general procedure was similar.

- (a) A range of standard catecholamine solutions appropriate to the biological samples being assayed was prepared (in the case of plasma catecholamines these were usually between 1 and 20 ng ml⁻¹) from the 100 µg ml⁻¹ stock solutions. The assay reagents were removed from the freezer, allowed to thaw and all reagents, standards and samples filtered (0.45 µm pore size) to remove particulate matter. Three different filters were used for the reagents, samples and standards and between each solution the filter was thoroughly washed with distilled water. A set of washed dust-free polypropylene tubes was used to receive the filtered solutions and similarly the reaction was carried out in washed dust-free tubes.
- (b) Each sample was assayed in duplicate (with a blank) in 12 x 75 mm polypropylene tubes. The buffer was added to the tube followed by the sample then, in the volumes and at the exact times indicated in Tables 4-4 and 4-5, the oxidant, the alkaline sulphite solution and the glacial acetic acid were added.

Table 4-4

Reagent Volumes (µl) used in the Fluorimetric Assay of the catecholamines

Assay	Buffer	Sample	Iodine	Alkaline Sulphite	Acetic Acid	Total
1-dopa	50	100	10	50	50	260
dopamine	50	100	10	50	50	260
noradrenaline	50	100	10	50	20	230
adrenaline	50	100	10	50	20	230

Table 4-5

Reaction Conditions used in the Fluorimetric Assay of the
Catecholamines

	L-dopa	Dopamine	Noradrenaline	Adrenaline
Buffer pH	5.4	7.0	6.5	4.0
Oxidation Time (minutes)	3	3	3	3
Tautomerization Time (minutes)	5	5	5	1
Final pH	5.7	4.4	4.8	5.0
Development Temperature ^o	100	100	20	20
Development Time (minutes)	40	40	25	1
Excitation Wavelength (nm)	330	320	380	410
Emission Wavelength (nm)	380	375	470	500

[from Laverty & Taylor 1968]

The test tubes containing l-dopa and dopamine samples were capped and transferred to a water bath maintained at 100^o. Development of the fluorophore occurred over 40 minutes at this temperature following which the solutions were allowed to cool to room temperature and the fluorescence determined (at the fluorimeter settings of Table 4-6). The contents of the adrenaline assay tubes were transferred directly to the fluorimeter cuvettes after adding the acetic acid and the fluorescence determined within 1-4 minutes. The noradrenaline fluorophore on the other hand required 25 minutes for maximum development. While a slow decomposition of the adrenaline fluorophore occurred within a few minutes of its development the remaining fluorophores were more stable and the timing of the fluorescence

Table 4-6

Fluorimeter (Aminco-Bowman Spectrophotofluorometer) Settings
used in the Fluorimetric Assay of the Catecholamines

excitation slit widths	1,1 (mm)
emission slit widths	5,5 (mm)
response	3
sensitivity	0.3-10

determination not as important. The timing sequence of the noradrenaline and adrenaline assays are such that if both are being analyzed in the same sample, as was usually the case, then the 2 assays may be intermeshed. In this manner it was possible for 2 people - one carrying out the assay (in groups of 3 samples and a standard) and the other determining the fluorescence, to assay 50 samples in a working day. In practice 1-dopa and dopamine are not assayed together because 1-dopa is usually present in insignificant amounts in biological material.

- (c) For each assay series a calibration curve was prepared to check for linearity together with an analysis of the interference by adrenaline in the noradrenaline assay and vice versa (Table 4-7) to allow for day-to-day variation in cross interference. 0.1 ml of the extracted eluate was added to a liquid scintillation vial and mixed with 10 ml of the scintillation cocktail. The vials were kept for 24 hours in the dark and counted with a Packard Tricarb 2425 liquid scintillation counter.

Volumes of the Assay Solutions The microassay described uses only $\frac{1}{5}$ of the sample volume used in the Laverty & Taylor (1968) method. Furthermore, a 50% increase in assay sensitivity has been achieved by increasing the sample to reagent volume ratio.

Cleanliness It must be emphasized that to measure blood catecholamines fluorimetrically meticulous attention to detail is essential. One of the most important sources of error is the presence of dust and particulate matter in the sample or reagent solutions. Because the fluorimeter is measuring at high sensitivity light scattering in the cuvette greatly increases the photomultiplier output. Thus the filtration of all solutions was of considerable importance in obtaining consistent duplicates and low blanks. In tandem with this precaution, all test tubes used were carefully washed with distilled water and dried to reduce the possibility of test tube contaminants being introduced to the assay system. Soluble contaminants, such as detergents, cause marked increases in blank levels which may obliterate the small catecholamine fluorescence signal.

Blanks None of the blanks described in the literature can be regarded as completely satisfactory, most giving values higher than might be expected from a "true blank". Four different types of blanks were investigated, the last described proving the most satisfactory and being adopted for general use in this work.

1. Faded blanks where the fluorophore is destroyed by heat or short-wave length irradiation.
2. Unoxidized blanks where iodine is omitted but the other reagents are added in the normal sequence.
3. Reversed blanks where reagents and sample are added together in the reverse order to the normal assay procedure.
4. A modified reverse blank where the reagents are added in the reverse order but omitting the iodine - the sample is added last.

Fluorimeter The choice of the fluorimeter in the assay of plasma catecholamines has an important bearing on the final reliability (§3.6). The ellipsoidal off-axis condensing system of the Aminco-Bowman Spectrophotofluorometer is claimed to increase sensitivity by a factor of 10 compared with conventional mirror systems for collecting the excitation radiation. Although the sample to blank ratio remains unaltered the slit widths may be reduced and resolution increased to improve discrimination. As important as sensitivity is the stability of the excitation radiation. With the highly amplified photomultiplier output necessary for the assay of catecholamines any small shift in the xenon lamp arc or fluctuations in its intensity seriously compromise the assay reliability. In the present case this was minimized in 3 ways: the use of a ratio photometer which monitors and corrects (at least partially) for source fluctuations, the installation of a magnetic arc stabilizer (which by creating a fluctuating magnetic field and inducing a small circular motion in the arc, constrains arc shifts) and the use of a standard solution to determine instrument stability throughout the assay of a series of unknowns.

Reliability of the Catecholamine Assay

Accuracy The losses of the catecholamine content of plasma during protein precipitation and extraction are of the order of 50% (§4.3). However the question of assay accuracy deserves further attention in view of the uncertain nature of the protein binding of these substances in plasma (§3.2). The range of values reported for noradrenaline and adrenaline in the plasma of resting mammals is wide. The lowest reported values may be influenced by unknown losses (many methods do not allow for losses in protein precipitation and extraction) and higher values by interference in the assay or, in many instances, by sampling disturbances to the animal (§5.2).

Specificity No investigation of possible interference in the fluorimetric assay of catecholamines has been made in this work, apart from the cross reactions between the various catecholamines. Instead reliance has been placed on the extensive investigations by other authors (Laverty & Taylor 1968, Griffiths *et al.* 1970). The substances that these investigators have shown to affect the catecholamine assay significantly are either not present in appreciable quantities in sheep plasma or are eliminated by the aluminium oxide extraction. It has however been noted that badly haemolyzed blood samples have on occasion given rise to high blanks. Incomplete protein precipitation or insufficient centrifuging of precipitated plasma samples are also potential sources of error.

The cross interference between the different catecholamines has been ascertained by measuring the fluorimetric responses of standard solutions of each compound ($1 \mu\text{g ml}^{-1}$) (the mean of 3 sets of duplicates) under the conditions of all 4 differential assays (Table 4-7).

Table 4-7
Cross-interference of the Catecholamines in
the Fluorimetric Assay (%)

	L-dopa	Dopamine	Noradrenaline	Adrenaline
l-dopa assay	100.0	3.1	0.0	0.0
dopamine assay	70.8	100.0	0.6	0.2
noradrenaline assay	0.2	0.3	100.0	6.9
adrenaline assay	0.2	0.3	2.7	100.0

The only interference of serious proportions is that by l-dopa in the dopamine assay. This level of cross-reactivity (70.8%) is such that the method is unsuitable for the determination of dopamine in the presence of appreciable amounts of l-dopa (a situation that does not occur naturally but is described in §5.3

during l-dopa infusions). Chromatographic separation is indicated in these circumstances. The other interactions are sufficiently small to be eliminated by mathematical means (Appendix III).

Calibration Curves

Eleven replicates of each of a series of standard solutions were determined for l-dopa, dopamine, noradrenaline and adrenaline. The resulting data were fitted to a linear equation.

$$y = ax + b$$

(where y is the fluorimetric responses, x is the standard solution concentration, a is the slope (coefficient of regression) and b is the intercept)

and to a quadratic equation

$$y = ax^2 + bx + c$$

by the method of least squares. In addition linear and quadratic functions were fitted to 11 individual calibration curve data sets obtained on 11 separate days.

A significant curvature was found in the l-dopa and dopamine calibration curves (Figure 4-4a,b) such that a quadratic equation gives a slightly better fit to the data than a linear equation. However from a practical point of view the curvature is small enough to ignore and as a first approximation a linear relationship was used in the assay of plasma samples. The calibration curves of noradrenaline and adrenaline are best described in terms of a linear equation, the quadratic giving no significant improvement in the fit over the assay ranges (Figure 4-4c,d). However if higher catecholamine quantities (>2 ng) are included in the assay data a significant quadratic component may be required to describe the curve. A highly significant ($P < 0.001$) difference in the slopes of the calibration curves of noradrenaline and adrenaline was found between the 11 different days. This reflects, in the main, changes in instrumental sensitivity on a day to day basis.

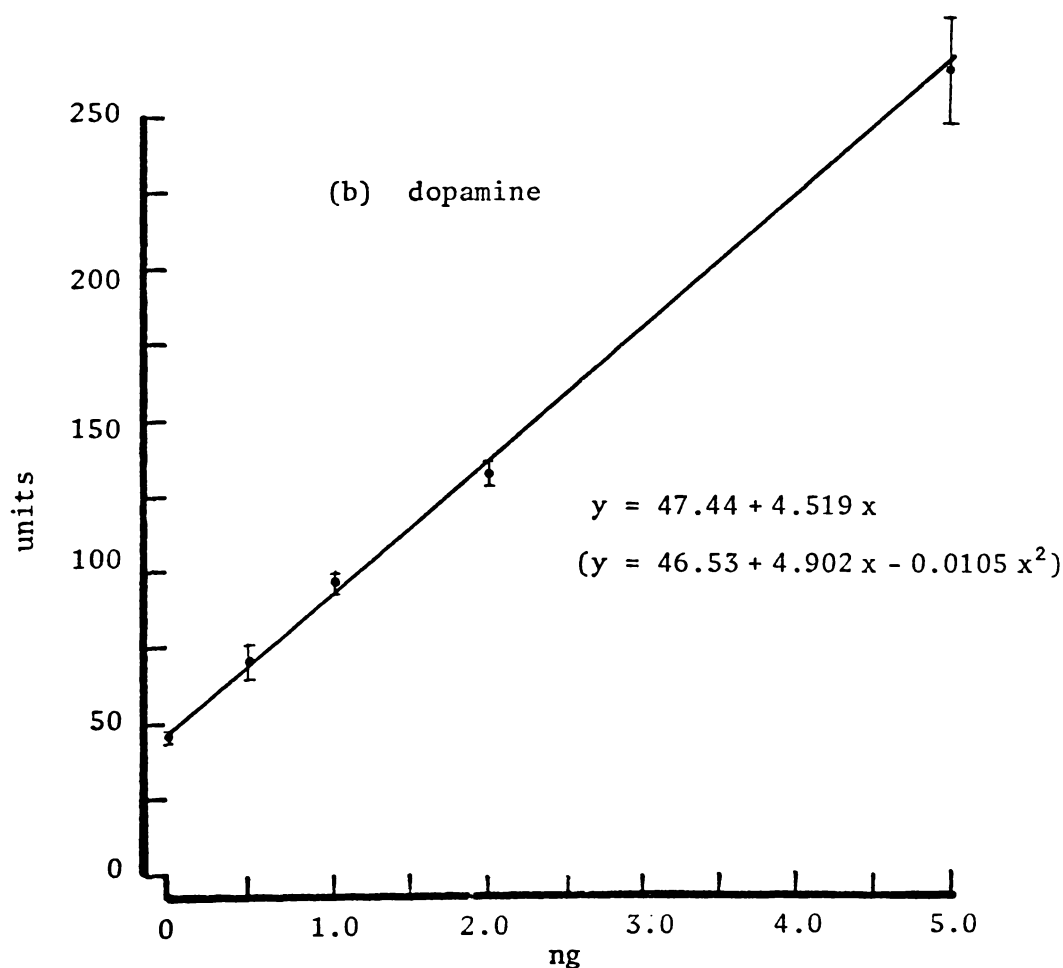
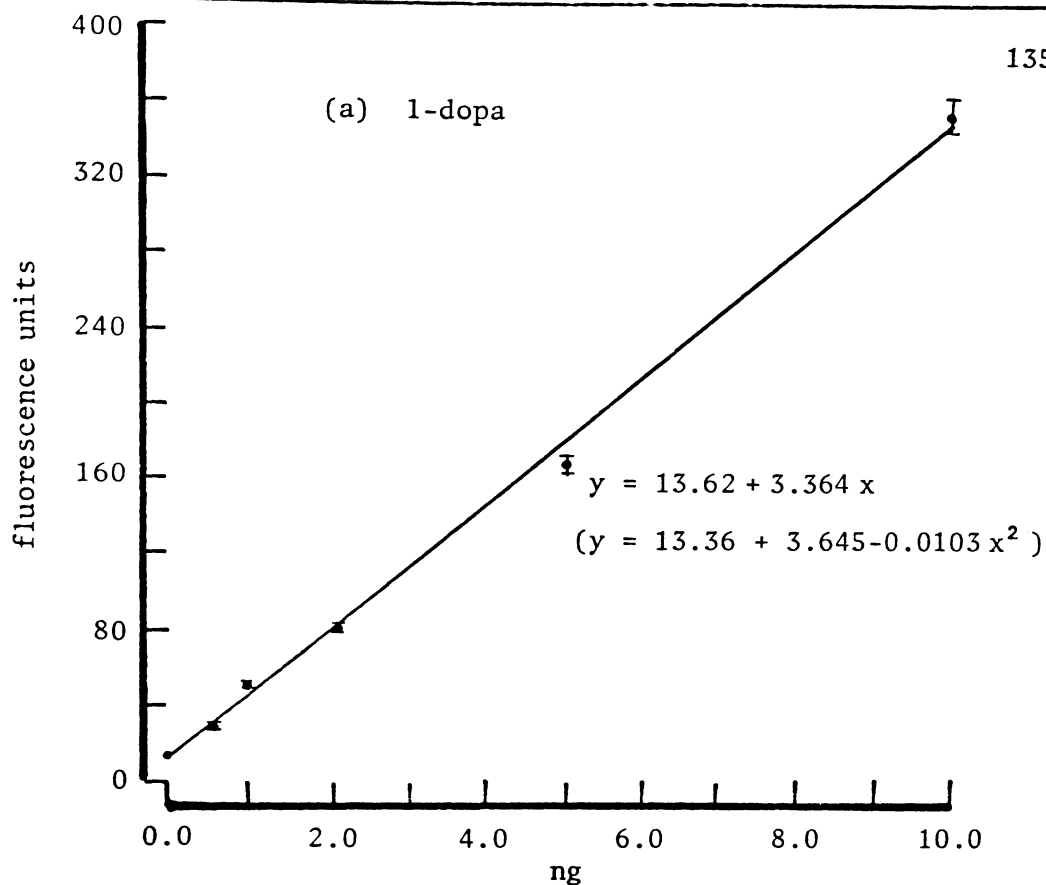


Figure 4-4(a)(b). L-dopa and dopamine standard solution calibration curves fitted by method of least squares. A small but significant curvature was found to be present and the fitted quadratic curve is given in brackets.
Experimental mean $\pm$ SD (n=11).

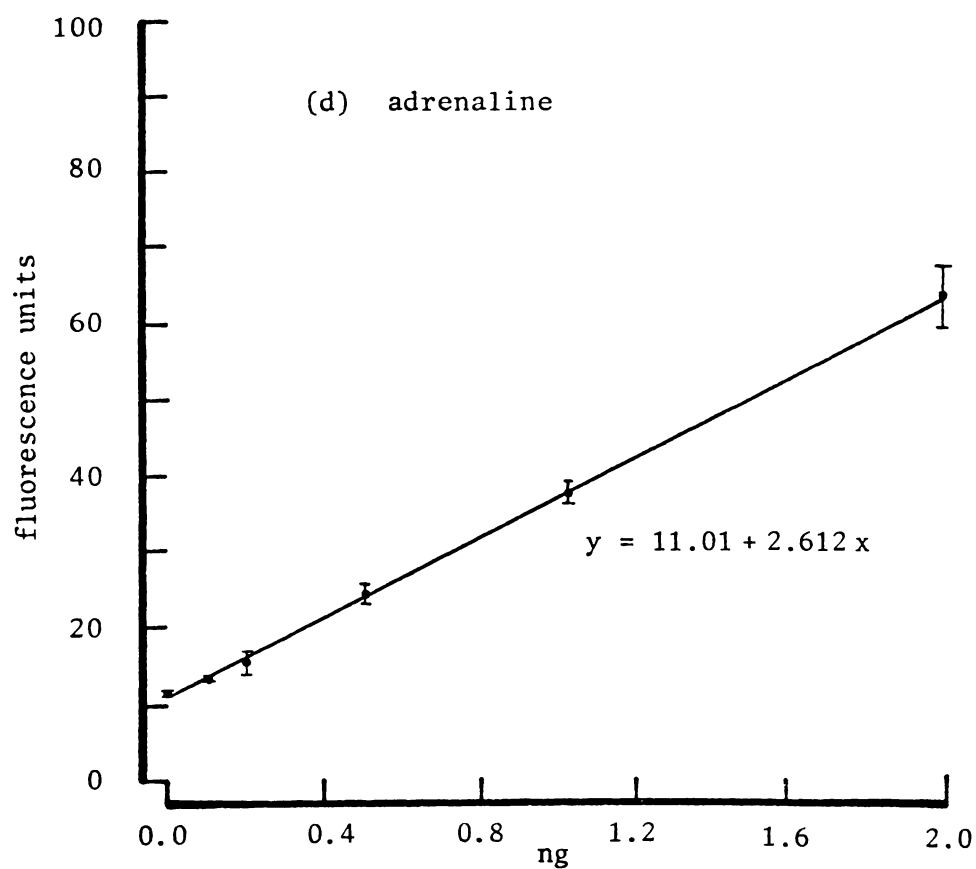
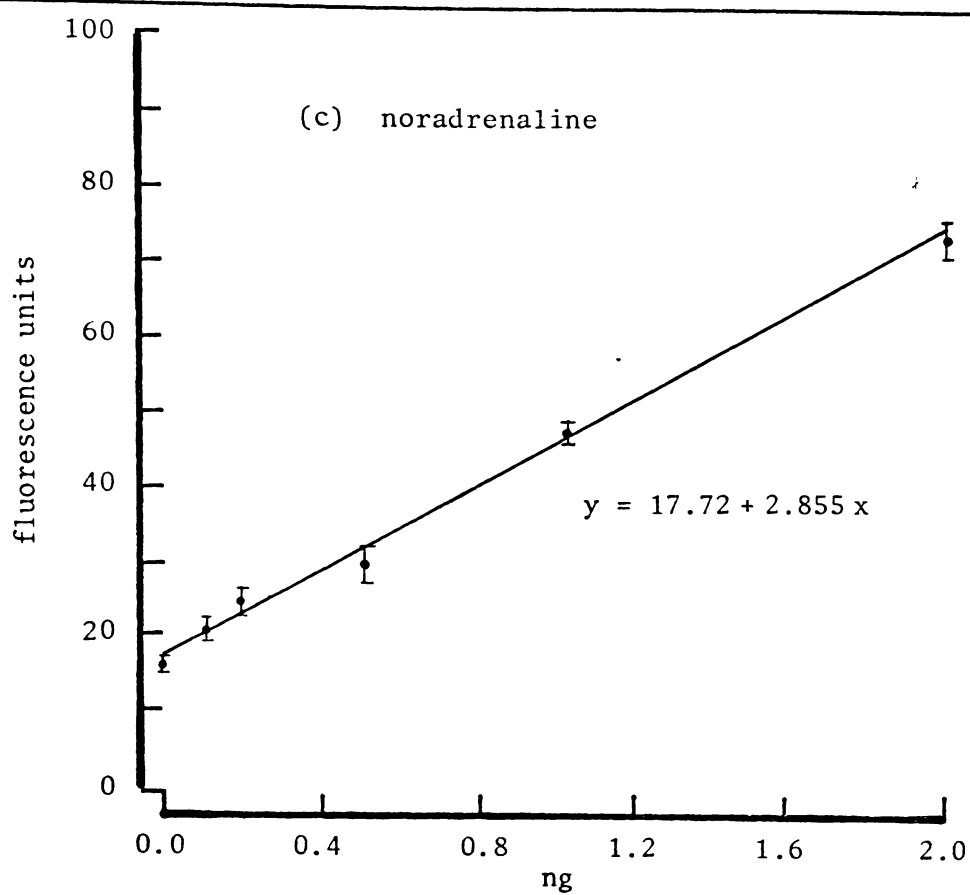


Figure 4-4(c)(d). Noradrenaline and adrenaline standard solution calibration curves fitted by method of least squares.

• Experimental mean $\pm$ SD (n=11).

A specific purpose FORTRAN IV computer programme (RXPC) was developed to integrate the recovery and the fluorimetric data and to calculate the plasma catecholamine concentrations. An outline of the programme structure and the necessary details for its use are presented in Appendix III.

4.5 RADIOENZYMATIC ASSAY OF CATECHOLAMINES

The radioenzymatic assay (§3.10) as described in the literature up until about 1976-1977 appeared to offer no real advantage over the fluorimetric procedure. This impression has been confirmed by the study of Miura *et al.* (1977). These investigators compared the fluorimetric assay of Renzini *et al.* (1970) with the double isotopic radioenzymatic assay of Engelman & Portnoy (1970), as modified by DeQuattro & Chan (1972), and concluded that both methods were of comparable sensitivity but that the radioenzymatic method was conspicuously more time consuming and complex. Yet since 1972 the sensitivity of the radioenzymatic procedure has greatly improved and the method simplified. For instance 1 ng of adrenaline yielded 44 counts per minute above the blank according to Engelman & Portnoy (1970). The same quantity of adrenaline produced 36 096 counts per minute in the assay of Peuler & Johnson (1977) and 211 149 counts per minute in that of Saller & Zigmond (1978). At the same time the volume of plasma required has fallen from 5-10 ml to as low as 10 μ l.

Analytical Methods

Development work in the present study was based initially on the method of da Prada & Zürcher (1976) but was later switched to the improved method of Peuler & Johnson (1977). No plasma protein precipitation is necessary and the reaction is carried out using 50 μ l of plasma. The ultimate sensitivity of the method is reported as 6 pg for dopamine and 1 pg for noradrenaline and adrenaline. Essentially the method relies on the conversion of the catecholamines into tritiated methoxy derivatives by the enzyme catechol-O-methyltransferase (§2.4) (Figure 4-5).

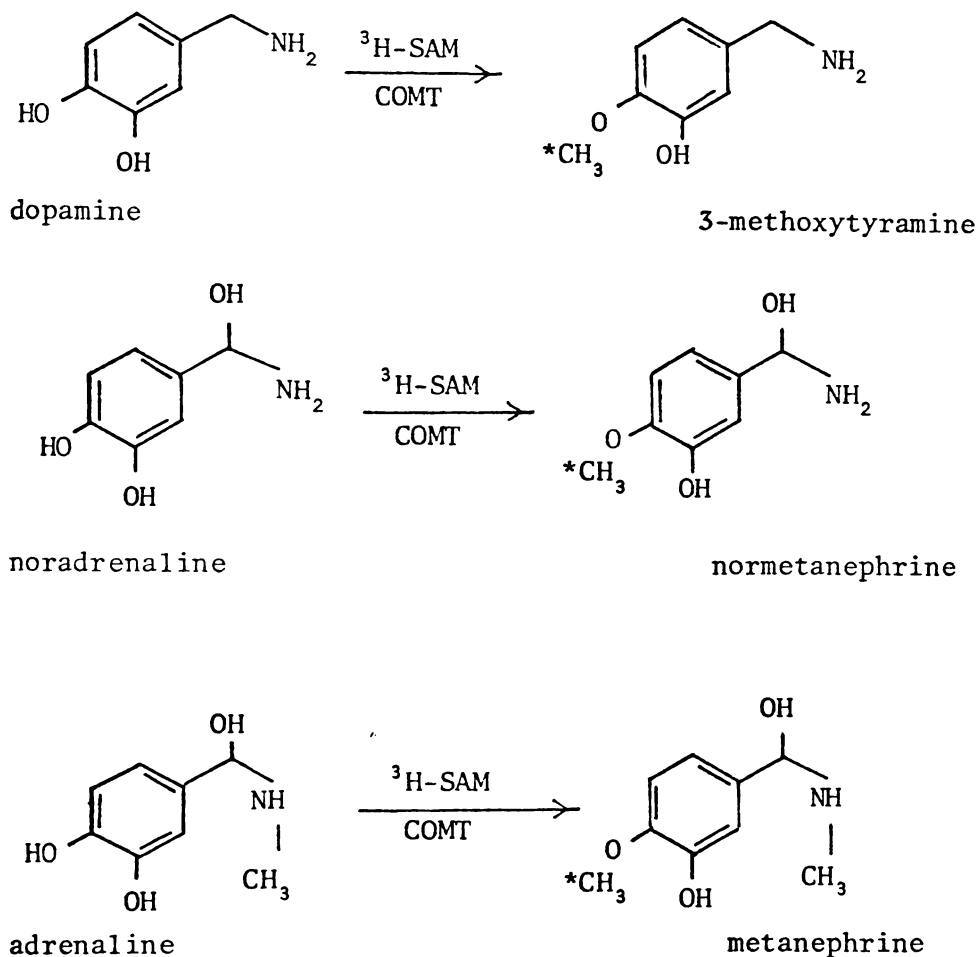


Figure 4-5. Radioenzymatic assay reactions.

$^3\text{H-SAM}$ $^3\text{H-S-adenosylmethione}$ $^*\text{CH}_3$ tritiated methyl group
 COMT catechol-O-methyltransferase

The methoxy derivatives of the 3 catecholamines are isolated by organic extraction, separated from each other on thin layer plates and the radioactivity counted by liquid scintillation.

Outline of the Steps in the Radioenzymatic Assay

50 μ l of plasma is incubated for 1 hr at 37° in a medium including ^3H -S-adenosylmethionine and catechol-O-methyltransferase



the reaction is terminated chemically, a quantity of the methoxy derivatives of all 3 catecholamines added and the incubated medium extracted in organic solvents with vigorous shaking



the aqueous layer is discarded and the organic phase extracted with dilute acetic acid (100 μ l)



the organic supernatant is discarded and the acetic acid washed with fresh organic solvents



150 μ l of ethanol is mixed with the acetic acid extract and the solution is applied to silica gel thin layer plates; the plates are developed and dried



the spots on the plates representing normetanephrine, metanephrine and 3-methoxytyramine are visualized under ultra-violet light, scraped off the plate into separate vessels and extracted into an ammonium hydroxide solution



normetanephrine and metanephrine are oxidized to vanillin, extracted into a toluene-based liquid scintillation cocktail and the vials are counted in a liquid scintillation spectrometer; 3-methoxytyramine is extracted into an organic solvent, the aqueous layer discarded, liquid scintillation cocktail added and the vials counted

Catechol-O-methyltransferase was initially prepared from rat livers as described by Axelrod & Tomchick (1958). However the activity of this preparation was significantly less than the enzyme that has recently become commercially available

(Sigma Chemical Company, St Louis, Missouri, USA). The optimum amount of this commercial enzyme per assay tube was determined to be 25 units using 2.5 μCi of S-adenosylmethionine. A unit of catechol-O-methyltransferase is defined as that amount that will catalyze the methylation of 1.0 mmole of protocatechuic acid (3,4-dihydroxybenzoic acid) per hour (pH 7.9, 37⁰) using ¹⁴C-S-adenosylmethionine as the methyl donor.

Results

The results of the replicate determinations of standard solutions of the 3 catecholamines (Table 4-8) indicate the linearity and specificity of the assay using 25 units of catechol-O-methyltransferase and 2.5 μCi of ³H-S-adenosylmethionine (11.4 Ci mmole⁻¹) per assay tube.

Table 4-8

Sensitivity and Specificity of the Radioenzymatic Assay
dpm $\pm$ SD (n=6)

Assayed Solution	3-methoxytyramine	normetanephrine	metanephrine
Blank	604 $\pm$ 42	578 $\pm$ 43	522 $\pm$ 38
Dopamine (1 ng)	30 250 $\pm$ 3 412	805 $\pm$ 48	1 232 $\pm$ 1 340
(10 ng)	306 322 $\pm$ 17 325	1 531 $\pm$ 162	10 261 $\pm$ 1 821
Noradrenaline (1 ng)	1 532 $\pm$ 158	10 203 $\pm$ 1 829	857 $\pm$ 58
(10 ng)	9 631 $\pm$ 2 457	113 782 $\pm$ 10 321	10 043 $\pm$ 2 190
Adrenaline (1 ng)	2 956 $\pm$ 702	859 $\pm$ 89	12 979 $\pm$ 1 261
(10 ng)	25 421 $\pm$ 4 341	5 434 $\pm$ 1 397	124 325 $\pm$ 7 560

dpm (disintegrations per minute)

The major remaining problems in the assay are related to the chromatography steps. In the present case recoveries of the tritiated derivatives dropped by about 50% in the thin-layer chromatography. The cross interference between catecholamines ranged up to 10% which is higher than the 0.1-1.8% reported by Peuler & Johnson (1977). Also hand spotting of 250 μ l of extract onto a thin layer plate is time consuming and it was difficult to achieve small uniform spots. The samples were transferred to the plate very much faster using a custom built automatic spotter but the flow rate was uneven and the spot sizes varied. High performance liquid chromatography seems to offer a superior alternative to thin layer chromatography both in terms of speed and separation efficiency and is currently being investigated.

Very high specific activity (up to 85 Ci mmole⁻¹) ³H-S-adenosyl-methionine has recently become available and if used in the present procedure would permit a further 5-10 fold increase in the assay sensitivity. It may be shown in fact that the theoretical maximum disintegration rate assuming complete conversion and recovery of 1 ng of adrenaline with 85 Ci mmole⁻¹ ³H-S-adenosylmethionine is 1.03×10^6 dpm.

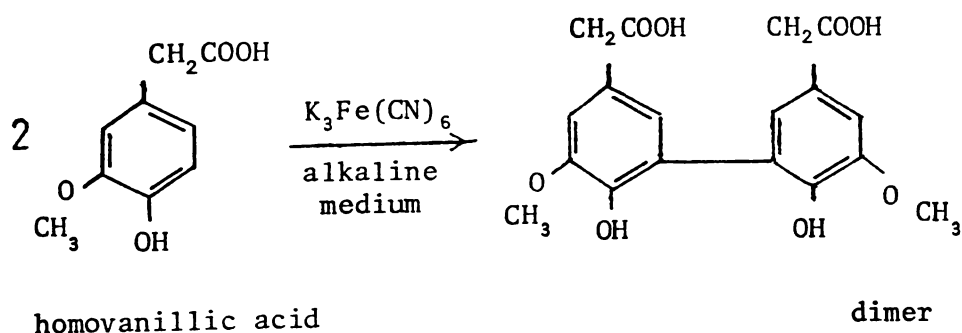
4.6 THE ANALYSIS OF HOMOVANILLIC ACID

The fluorimetric assay of cerebrospinal fluid homovanillic acid (3-methoxy-4-hydroxyphenylacetic acid) is briefly described here; for fuller details the reader is referred to the papers of Andén *et al.* (1963), Korf *et al.* (1971) and Westerink & Korf (1975) on which the present methodology is based. Alternative methods of assay are with gas-liquid chromatography (electron capture detection) (Dziedzic & Gitlow 1974, Watson & Wilk 1975) and mass fragmentography combined with gas-liquid chromatographic separation (Karoum *et al.* 1975, Carl-Gunnar *et al.* 1976). Whilst these methods offer advantages over fluorimetric methods in specificity

and sensitivity, the additional chemical conversions and the specialized equipment necessary have to be balanced against the convenience of the fluorimetric assay.

Solvent extraction, most commonly with diethyl ether (Carl-Gunnar *et al.* 1976) or ethyl acetate (Gerbode & Bowers 1968), is used to separate homovanillic acid from other substances in cerebrospinal fluid. In the present study redistilled diethyl ether was found to give more consistent and lower blanks. Using the method of Gerbode & Bowers (1968), including preliminary protein precipitation with zinc sulphate and sodium hydroxide, recoveries of homovanillic acid added to cerebrospinal fluid were of the order of 65-75%. However the 0.5 M phosphate buffer eluant adopted by Gerbode & Bowers (1968) quenched the sample fluorescence and was replaced by 0.05 M tris buffer (pH 8.5). Westerink & Korf (1975) also encountered quenching associated with the use of phosphate buffer.

The fluorimetric assay of homovanillic acid in its various modifications is based on the discovery by Andén *et al.* (1963) that a strongly fluorescent compound was formed by homovanillic acid when oxidized in an alkaline solution. Subsequently the fluorophore was isolated and characterized by Corrodi & Werdinius (1965) as a dimer.



Equipment

Millipore filters (0.45 μm)
polypropylene test tubes (12 x 75 mm, 15 x 100 mm)
Eppendorf piston pipettes
Aminco-Bowman Spectrophotofluorometer
Quartz cuvettes (7 x 7 x 32 mm)
Sargent-Welch pen recorder

Solutions

homovanillic acid (100 $\mu\text{g ml}^{-1}$)

10 mg of homovanillic acid was dissolved in 100 ml of 0.001 N HCl and dilutions made daily in 0.05 M tris buffer (pH 8.5).

cysteine (0.4% w/v)

52 mg of cysteine hydrochloride was dissolved in 10 ml of water. This solution was discarded after 3 hours at room temperature.

potassium ferricyanide (0.1% w/v) - ammonia (2 N)

10 mg of $\text{K}_3\text{Fe}(\text{CN})_6$ was dissolved in 10 ml of 2 N ammonia, the day before use and stored at 4°. A blank reagent of 2 N ammonia alone was also prepared.

tris buffer (0.05 M)

1.514 g of 2-amino-2-hydroxymethyl-1,3-propanediol was dissolved in 150 ml of distilled water, the pH adjusted to 8.5 and the volume made up to 250 ml.

zinc sulphate (5% w/v)

8.90 g of $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ was dissolved in 100 ml of water.

Isolation of Homovanillic Acid from Cerebrospinal Fluid

After collection cerebrospinal fluid samples were stored frozen at -20°. Samples contaminated with blood were discarded. Three ml of cerebrospinal fluid was deproteinized with 1.0 ml of 5% ZnSO_4 and 0.5 ml of 1N NaOH. After centrifuging at 2000 g for 5 minutes the supernatant was added to a 15 x 100 mm polypropylene test tube along with 1.0 ml of 1 N HCl, 3 g of NaCl and 5 ml of water-washed redistilled diethyl ether.

The test tube was capped, shaken for 15 minutes and centrifuged at 1000 g for 5 minutes. 4.5 ml of the separated organic phase was transferred to a second polypropylene test tube containing 2.0 ml of 0.05 M tris buffer pH 8.5. The test tubes were capped, shaken and centrifuged as before.

Assay

Polypropylene test tubes (12 x 75 mm), washed and dried before use, held the filtered assay solutions. Triplicates of each sample together with duplicate blanks were assayed in parallel. Blanks were prepared by adding the reagents in the reverse order with the sample added last. The ammonia solution used in the blank contained no $K_3Fe(CN)_6$. Samples were assayed by adding 0.02 ml of the $K_3Fe(CN)_6$ -ammonia solution to 0.2 ml of the unknown followed by 0.02 ml of cysteine (0.4%) after exactly 2 minutes. The fluorescence developed was relatively stable but was always read within a few minutes (excitation 310 nm and emission 420 nm). The mean blank was subtracted from the mean sample peak height and the homovanillic acid concentration calculated from a calibration curve constructed using extracted standard solutions of homovanillic acid.

Reliability of the Homovanillic Acid Assay

As with the catecholamine assay (§4.4) glassware cleanliness and reagent purity are considerations of greatest importance. All solutions, standards and samples were filtered through 0.45 μ m pore diameter filters.

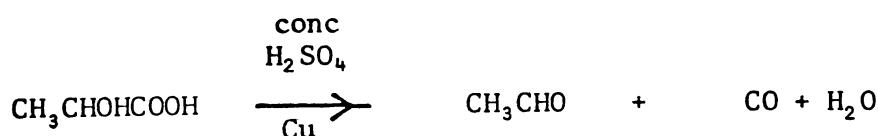
The fluorimetric method for homovanillic acid determination has come under increasing criticism in recent years on the grounds of specificity (Kirschberg *et al.* 1972) and sensitivity (Dziedzic & Gitlow 1974). Yet the assay employed in the present work has evolved from the earlier assay methods with considerable improvements in sensitivity (Westerink & Korf 1975). Furthermore most of the acid metabolites found to suppress fluorophore development in the study of Kirschberg *et al.* (1972) are not likely to be

present in sheep cerebrospinal fluid to a degree sufficient to cause significant distortions.

Above 20 ng ml^{-1} the calibration curve was linear, below this level the response was curvilinear and less than expected from the extrapolation of the linear component. This phenomenon has been reported previously by Westerink & Korf (1975). It is clear that the measurement of homovanillic acid in cerebrospinal fluid ($<50 \text{ ng ml}^{-1}$) is at the limits of the assay sensitivity and further methods must be developed for the study of small changes in homovanillic acid in this concentration range.

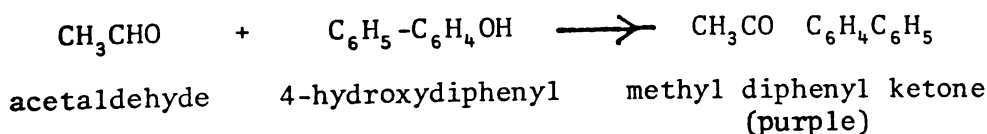
4.7 THE ANALYSIS OF LACTIC ACID

Lactic acid, a product of carbohydrate metabolism, is commonly assayed by 3 principal procedures; namely chemical, enzymatic and gas chromatographic. The chemical procedure is based on the chemical conversion of lactic acid to acetaldehyde (by heating in concentrated sulphuric acid, catalyzed by copper) which is subsequently coupled to an aromatic compound, 4-hydroxydiphenyl to produce a purple product measured spectrophotometrically (Barker & Summerson 1941).

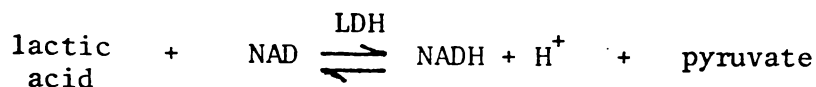


lactic acid

acetaldehyde



Enzymatic methods, now in widespread use, depend on the following reaction.



NAD nicotinamide adenine dinucleotide LDH lactate dehydrogenase

By measuring the change in the NADH concentration either spectrophotometrically (Neville & Gelder 1971, Everse 1975) or fluorimetrically (Olsen 1971) the original lactic acid content of the sample may be calculated. By forcing the reaction from right to left pyruvate may also be determined. The enzymatic method while more specific and more rapid than the chemical route requires careful control of temperature, pH and timing. Chemical procedures although more sensitive and hence requiring smaller blood samples are more time consuming and require meticulous attention to detail.

The specific variation of the Barker-Summerson method of chemical analysis used in this work (Pryce 1969) is less time consuming and at the same time a more sensitive assay. The carbohydrate elimination step of the original method is dispensed with since interference by glucose is small and unlikely to be significant in most circumstances. The concentration of copper, which accelerates the conversion of lactic acid to acetaldehyde is increased in the interest of a more rapid reaction, while the p-hydroxybiphenyl is dissolved in dimethylformamide instead of alkali as the resulting reagent is stable over long periods of time and mixes more easily. The sensitivity of the method is also enhanced by the addition of phosphoric acid. Only 50 μl of plasma is required.

Blood collected for lactic acid determination must be processed rapidly to prevent changes in its lactate (and pyruvate) concentrations due to glycolytic erythrocyte activity (Huckabee 1956). However Maguire & Copeland (1972) found no significant difference

in lactic acid concentration of blood samples left for 15 minutes compared to those treated immediately to obtain protein-free filtrates but a 5 mg dl^{-1} increase in the lactic acid of whole blood samples was evident at 60 minutes post-collection. It is noted in this connection that a mixture of 10 mg sodium fluoride and 2 mg of potassium oxalate per ml of whole blood is adequate to inhibit glycolysis (and clotting) for 24 hours at 4° (Savory & Kaplan 1966).

In the present study plasma for both cortisol and lactic acid determinations was separated from whole blood by centrifuging usually within 15 minutes, and not longer than 30 minutes, after drawing the blood. In addition the sample was cooled in a refrigerated water bath to depress metabolic processes prior to centrifugation. Heparin, used as the anticoagulant (§4.4), has no effect on the assay (Maguire & Copeland 1972). The plasma was stored at -20° until analysis.

Equipment

MSE Minor benchtop centrifuge (Measuring and Scientific Equipment Ltd, Crawley, UK).

15 x 150 mm Pyrex test tubes

Unicam SP 600 Spectrophotometer (Unicam Instruments Ltd, Cambridge, UK).

test tube mixer (Lab-Line Instruments Inc, Melrose Park, Illinois, USA).

boiling water bath.

Assay Solutions

lactic acid

standard solutions were made by adding 213 mg of lithium lactate to 50 ml of water followed by 0.5 ml of concentrated sulphuric acid. The resulting solution was diluted to 500 ml with distilled water to give a final concentration of 40 mg dl^{-1} . Further dilutions were then made representing 8, 16, 24, 32 mg dl^{-1} .

plasma deproteinizing solution

to 50 g of trichloroacetic acid in 500 ml of distilled water was added 22 ml of orthophosphoric acid and 5 g of cupric sulphate and the solution made up to 1 l with distilled water.

4-hydroxydiphenyl

2 g of 4-hydroxydiphenyl were dissolved in 100 ml of dimethylformamide.

Analytical Method

- (a) 0.1 ml of plasma was added to 4 ml of the trichloroacetic acid solution in a 10 ml conical glass test tube, mixed and centrifuged at 2000 g for 5 minutes.
- (b) 2 x 1 ml supernatant aliquots were transferred to 15 x 150 mm Pyrex test tubes and rapidly mixed with 6 ml of concentrated sulphuric acid added from an automatic burette. The test tubes were immediately placed in a boiling water bath for 5 minutes, removed and cooled rapidly in an ice bath. Up to 34 plasma samples in duplicate plus a duplicate set of standards (to construct a calibration curve) and duplicate blanks were assayed together.
- (c) 0.1 ml of 4-hydroxydiphenyl solution was added to each test tube, mixed and left to stand for 5 minutes, mixed again and left for 15 minutes. The test tubes were heated in the water bath for exactly 90 seconds to destroy excess 4-hydroxydiphenyl and cooled.
- (d) A portion of the solution was transferred to 10 x 10 mm cuvettes and the absorbance determined at 565 nm. A calibration curve was constructed from the standards between 0 and 40 mg dl⁻¹ and the lactic acid content of the unknowns calculated by simple proportionality after blank subtraction and averaging of duplicates. Samples containing lactic acid in greater concentration than 40 mg dl⁻¹ were diluted and reassayed.

General Procedure Thorough mixing of reagents at the various stages of the assay is essential and care should be taken to ensure uniform heating of the tubes in the boiling water bath. Since contamination by lactic acid in perspiration is possible (Barker & Summerson 1941, Harrower & Brown 1972) and resting values in sheep are $<5 \text{ mg dl}^{-1}$ (§5.4) erraneously high values from this source must be guarded against. Glassware was soaked between assays in detergent (chromic acid should be avoided) rinsed in distilled water and dried. The sensitivity of the assay was found to alter with different lots of sulphuric acid and new standards were necessary if a change was necessary during the determination of any series of unknown samples.

Reliability of the Lactic Acid Assay

Specificity Although there have been no indications in the current work that interfering substances are present to any significant degree, the reaction is not completely specific for lactic acid. Other hydroxy acids and aldehydes are potential sources of error (Barker & Summerson 1941). These however are unlikely to be present in sufficient concentrations to cause problems. Glucose interference amounts to only 1% of the colour developed by an equal weight of lactic acid (Pryce 1969).

Calibration Curves Eleven replicates of a series of standard solutions of lactic acid (0, 8, 16, 24, 32, 40 mg dl^{-1}) were determined in order to construct a calibration curve. In addition exogenous lactic acid was added (to a final added concentration of 0, 8, 16, 24, 32 and 40 mg dl^{-1}) to aliquots of a pooled plasma sample. Again 11 replicates of each plasma concentration were assayed. Finally the same plasmas were determined for lactic acid on 11 separate days.

Linear equations (derived by least squares fitting) described the calibration curves for the standard solutions and the plasma samples (Figure 4-6). Extending the curve to incorporate assay data from 100 mg dl^{-1} ml samples had the effect of producing a significant quadratic curvature into the calibration curve.

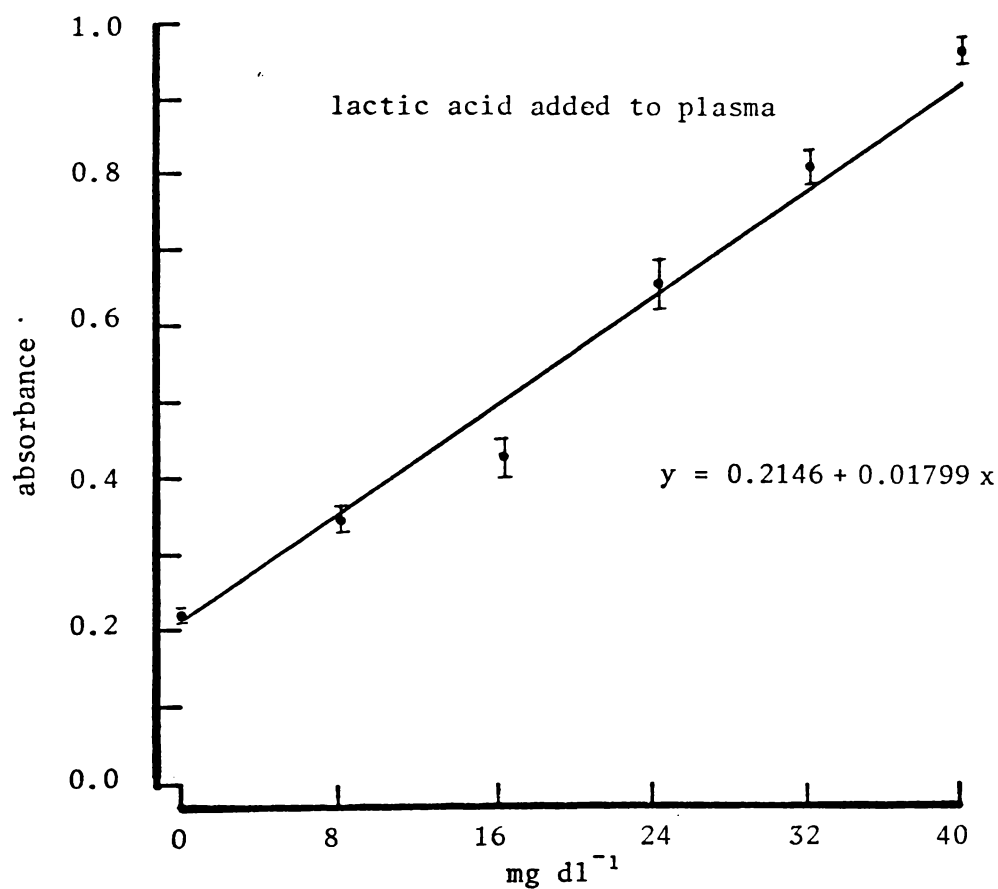
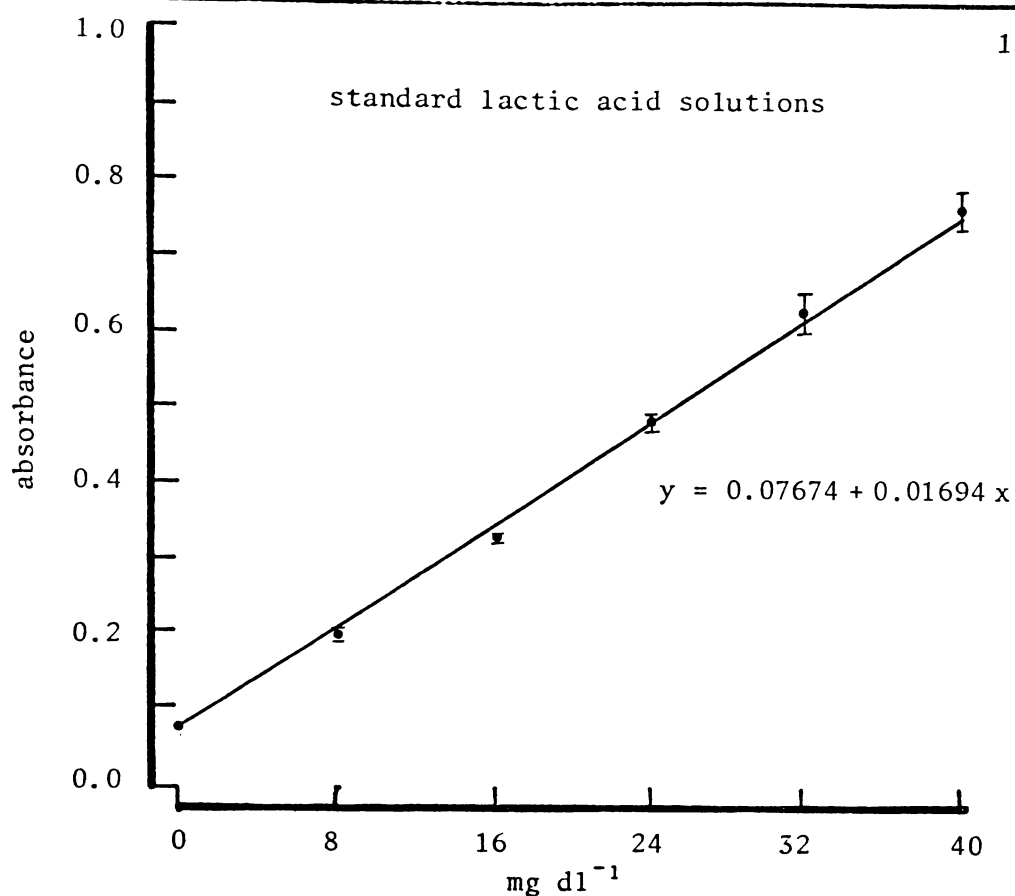


Figure 4-6. Lactic acid calibration curves fitted by the method of least squares.

Experimental mean $\pm$ SD (n=11).

No significant differences were found in the slopes or the intercepts of 11 calibration curves determined on separate occasions.

4.8 THE ANALYSIS OF CORTISOL

All plasma cortisol analyses were carried out by the Ruakura Chemical Services Section under the supervision of Dr R. Fairclough according to the methods of Fairclough *et al.* (1975). The procedure is an adaptation of the protein-binding assay of Murphy (1967) and is described in detail by Hudson (1975).

4.9 CONCLUSIONS

Progress in the study of blood catecholamines depends on their accurate determination. The fluorimetric method is adequate both in terms of sensitivity and precision for the physiological situations examined in this study. However small changes in the levels of blood catecholamines ($<0.2 \text{ mg ml}^{-1}$) are difficult to detect by fluorimetry without large blood volumes or replicate determinations. Minor fluctuations of blood-borne catecholamines occur under low level stimulation of many types (such as changes in posture, mild behavioural arousal, and drugs such as nicotine). These phenomena will become amenable to study with newer techniques such as the radioenzymatic assay whose ultimate sensitivity is limited only by the specific activity of the S-adenosylmethionine.

On the other hand technological advances in the study of fluorescence spectroscopy have already been made that could greatly improve both the sensitivity and precision obtained by fluorimetry. These advances are of 2 kinds - data processing and instrumentation. Virtually any measurement problem in fluorescence spectroscopy is complicated by alterations of the true signal by the instrumentation (such as light source instability, reabsorption of emission and photomultiplier wavelength

sensitivity). Digital processing can correct for these artefacts and significantly improve on the results obtained with electronic analogue circuitry (Wampler 1976). On the instrumental side the commercial availability of dye-tunable lasers (Latz 1976) and photon counting detection (Fitzgerald 1976) offer considerable improvements in sensitivity and resolution available over conventional equipment. The use of mathematical procedures to separate overlapping spectra (O'Haver 1976) could also be applicable to the catecholamine assay.

CATECHOLAMINE PLASMA DYNAMICS AND THE
EFFECTS OF INFUSED CATECHOLAMINES
ON LACTIC ACID AND CORTISOL

CHAPTER 5

5.1 INTRODUCTION

The problems in deciding the magnitude of the adrenergic response to any given challenge and relating this response to a particular physiological effect are not readily resolved because few studies of environmental and other stressors have included the simultaneous, detailed measurement of plasma catecholamines or estimates of the sympathetic discharge. Quite apart from the problems of assay (Chapter 3) there are other technical difficulties related to the physiological control of catecholamine secretion and transport that must also be considered. The literature covering these aspects is briefly reviewed together with an introduction to lactic acid and cortisol physiology relevant to the following experimental work. In the present chapter the experimental data described cover the dynamics of the catecholamines in ovine plasma, their endogenous release in certain situations and the effects of exogenous catecholamine infusions on plasma lactic acid and cortisol. Rapid serial blood sampling was carried out at the termination of each catecholamine infusion in order to estimate catecholamine half-lives in the sheep circulation. The cannulation procedures (§5.2) presented an opportunity to ascertain the effects of minor surgery on sheep under halothane anaesthesia and the time necessary for a return of plasma catecholamines, lactic acid and cortisol to baseline levels.

5.2 OVINE PLASMA CATECHOLAMINE LEVELS IN CANNULATION, RECOVERY AND DURING EXOGENOUS CATECHOLAMINE INFUSIONS

INTRODUCTION

Catecholamines in the Blood

The circulating concentration of the catecholamines represents what essentially remains after the interaction of a complex set of processes. As with all blood borne substances the concentration at any time is a balance between the amount released and the amount removed by tissue uptake, by metabolism and by excretion. The major considerations to be taken into account when interpreting data on plasma catecholamines are how the sample was obtained, which blood vessel was sampled and the history and the timing of stimuli promoting catecholamine release in the period preceding sampling (Callingham 1975, Callingham & Barrand 1976).

As assays have improved over the last 30 years so the estimated basal levels of catecholamines in the plasma have declined (§3.6). The best current estimates of dopamine, noradrenaline and adrenaline in the venous plasma of humans at rest are listed in Table 5-1.

In the cow Bühler *et al.* (1978) found very similar levels of the three catecholamines to those of humans. Generally it is clear that in all the species examined by Bühler *et al.* (1978) basal noradrenaline levels predominate over those of both dopamine and adrenaline. Dopamine is a normal plasma constituent of the resting animal and indeed exceeds the adrenaline concentration in some species. The amounts of circulating dopamine in sheep and goats (up to 0.4 ng ml^{-1}) (Kelly *et al.* 1970) appear similar to the amounts in human plasma.

Adrenaline in the blood is almost entirely of adrenal origin (Wurtman 1965, Roizen *et al.* 1974) and variations in its regional distribution reflect the relative uptake efficiencies of the perfused tissues. The sources of noradrenaline are the adrenal medulla (though the importance of this source will vary

Table 5-1

Dopamine, Noradrenaline and Adrenaline Concentrations
(ng ml⁻¹+SD) in the Venous Plasma of Normal Resting Human Subjects

DA	NA	A	Method	Reference
-	0.24 ±0.09	0.04 ±0.04	Fluorimetric	Griffiths <i>et al.</i> (1970)
0.20 ±0.06	-	-	Radioenzymatic	Christensen (1973)
-	0.18 ±0.06	0.04 ±0.03	Radioenzymatic	Cryer <i>et al.</i> (1974)
-	0.38 ±0.18	0.09 ±0.05	Fluorimetric	Diamant & Byers (1975)
-	0.33 ±0.05	0.39 ±0.04	GLC	Lovelady & Foster (1975)
0.31 ±0.24	-	0.15 ±0.08	GLC-mass spectrometer	Wang <i>et al.</i> (1975a)
0.034 ±0.031	0.279 ±0.169	0.023 ±0.019	Radioenzymatic	Peuler & Johnson (1977)
0.098 ±0.066	0.203 ±0.033	0.064 ±0.016	Radioenzymatic	Bühler <i>et al.</i> (1978)

from one species to another) and the post-ganglionic sympathetic nerve endings (Brown 1965, Sapira & Bron 1971). Changes in circulating noradrenaline are complex to interpret since it is not known what proportion of the noradrenaline is of sympathetic nervous origin. And since the proportion lost from the nerve endings is only a small proportion of the total released the overflow is very subject to alterations in reuptake efficiency or catabolizing enzyme activity. Plasma concentrations of noradrenaline may not therefore accurately reflect the quantitative changes in the sympathetic nervous system. Problems such as these emphasize the hazards of relying on plasma concentrations of noradrenaline as the sole index of sympathetic activity. Small amounts of noradrenaline and, to a much lesser extent, adrenaline may also originate from extramedullary chromaffin tissue, though the importance of this source is likely to be minimal excepting in

extra-adrenal phaeochromocytoma (Itoh & Ohmori 1973). The origin of plasma dopamine is not known for certain. Lishajko (1970) has demonstrated dopamine release by the isolated perfused sheep adrenal and tritiated dopamine release by the adrenal *in situ* (preloaded with ^3H -tyrosine) has also been observed in the ether-anaesthetized cat (Hempel & Männl 1967). Furthermore sympathetic discharge causes dopamine increments in the blood, albeit less than the rises in noradrenaline or adrenaline (Bühler *et al.* 1978).

After adrenalectomy adrenaline essentially disappears from the plasma (Price 1957) and urine (von Euler *et al.* 1954) of humans but noradrenaline remains. Adrenalectomy in the rat eliminates adrenaline from the plasma but dopamine, while reduced in concentration, does not disappear, suggesting an extra-adrenal origin for a significant portion (Bühler *et al.* 1978).

The catecholamines are rapidly removed from the circulation by re-uptake into nerve endings and by metabolic degradation (§2.4). As a consequence plasma half-lives are very short. The most important reason for the prompt decline in circulating catecholamines is the efficient uptake of these substances by the tissues (Iversen 1975). Vane (1966) found that over 90% of infused adrenaline (intra-aortic) is removed in one circulation through the cat hindquarters. The liver is an important sink for circulating catecholamines (Vane 1969) but the kidneys, gut and other organs and tissues are also important (Celander & Mellander 1955, Ginn & Vane 1968, Vane 1969). The lungs appear to remove significant quantities of noradrenaline without affecting the adrenaline content (Ginn & Vane 1968). This observation is of relevance to the present study where most blood samples were collected from the carotid artery.

As a consequence of the regional variation in catecholamine uptake significant differences in the content of blood drawn from different regions of the body occur. Vendsalu (1960) demonstrated that the lowest amounts of catecholamine were to be found in the hepatic vein and the highest from the vena cava and left

renal vein, attributable no doubt to adrenal proximity. Häggendal (1963b) also found very low catecholamine concentrations in the hepatic vein. Tissue uptake is believed to account for the adrenaline concentration gradient from venous to arterial blood (Vendsalu 1960). Roizen *et al.* (1975) found that total catecholamines in the arterial blood of rats was significantly higher than venous levels in a number of situations but combined blockade of extraneuronal and neuronal uptake reduced the difference. Since the noradrenaline concentrations did not account for the total catecholamine gradient most or all of the remaining difference can be attributed to adrenaline. On the other hand venous plasma is richer in noradrenaline than arterial plasma (Vendsalu 1960, Wallace *et al.* 1961) presumably owing to a net release of noradrenaline by peripheral tissue. In a more recent publication however Buhler *et al.* (1978) measured both higher noradrenaline and adrenaline in the carotid artery of the rat compared to the jugular vein. In view of the variability of the data the difference did not reach significance.

The nature of the release of catecholamines by the nervous system appears, even under resting conditions to be in the form of intermittent bursts (Sapira & Bron 1971). It may be mentioned in this context that in 60% of the human adrenal vein samples obtained by Sapira & Bron there was no detectable noradrenaline, the measured catecholamine being exclusively adrenaline. These findings together with the nature of tissue uptake explain the very transitory nature of plasma catecholamine levels. Frequent sampling and an understanding of the dynamics and dispersion of the catecholamines are therefore essential to an accurate conception of secretion rates over any period of time. Large individual variations in catecholamine responses under resting and stimulating conditions and marked divergences in the noradrenaline/adrenaline ratio of adrenal medullary output (Sapira & Bron 1971, Bühler *et al.* 1978) must also be taken into account.

Finally there remains the difficulty of obtaining blood samples without triggering sympathetic discharge. Many potential stressors involved in blood sampling particularly in non-conditioned domestic stock (pain, the unfamiliar personnel, procedure and surroundings) may make so called basal levels almost meaningless. Bühler *et al.* (1978) have examined this problem and shown that with an indwelling cannula and care in sampling their basal catecholamine levels were not only lower than in many other published experiments but also exhibited less variability. Gentle handling of rats for as short a time as 30 seconds caused rises in adrenaline of 1000%, noradrenaline of 300% and dopamine of 100%. Restraint stress caused even larger increments in catecholamine levels. In view of this and similar reports great care is essential in blood sampling. The stimulus-secretion time lag in cortisol secretion (§5.5) for example does not apply to catecholamine release and even the most rapid sampling without the use of cannulae is likely to produce artefacts.

Blood Catecholamines in Anaesthesia, Surgery and During Recovery

Previous studies of sympathetic nervous system participation in the physiological adaptations that occur in anaesthesia and surgery have come to inconsistent conclusions. Furthermore the direct effects of anaesthesia may be complicated by other factors such as acidaemia, hypoxia and changes in body temperature (Callingham 1975). Data gathered by Price (1959) indicated that in humans under diethyl ether anaesthesia, increases in adrenaline (estimated by the trihydroxyindole fluorimetric method) were smaller ($+0.082 \pm 0.084$ ng ml⁻¹, n=7) than in noradrenaline ($+1.50 \pm 0.71$ ng ml⁻¹, n=7). Price *et al.* (1959) reported no consistent changes in plasma catecholamines during halothane or thiopental anaesthesia but increases with diethyl ether and cyclopropane. Surgery, in addition to anaesthesia may increase circulating catecholamine levels (Hamelberg *et al.* 1960, Anton *et al.* 1964), more so when diethyl ether is employed than with halothane (Anton *et al.* 1964). Recently with the advent of the radioenzymatic assay it has become apparent that halothane significantly decreases catecholamine output (Roizen *et al.* 1974, Halter *et al.* 1977)

although surgery may subsequently cause catecholamine levels to rise (Halter *et al.* 1977). Halothane is a known depressant of cardiovascular (Eger *et al.* 1970) and neural function (Skovsted *et al.* 1969) and a fall in blood catecholamines is a not unexpected corollary. Adrenaline is decreased more than noradrenaline both in relative and absolute terms indicating a higher medullary sensitivity compared with the peripheral nervous system (Halter *et al.* 1977). Significant rises in both noradrenaline ($+0.38 \pm 0.17 \text{ ng ml}^{-1}$, $n=8$) and adrenaline ($+0.25 \pm 0.28 \text{ ng ml}^{-1}$, $n=8$) occurred and levels remained high for at least 2 hours following surgery (Halter *et al.* 1977). Since hypoxia, hypothermia or hypotension could not explain the sympathetic activation it was suggested that the findings were compatible with stimulation in response to afferent signals from the site of surgical trauma.

Catecholamine Infusion Studies

Many previous investigators have examined blood catecholamine levels in various species before, during and after catecholamine infusions and injections (Table 5-2). However the reported pre-infusion catecholamine levels in the plasma and the quantitative estimates of the catecholamine levels reached under similar or identical infusion regimes differ widely. Qualitatively, an initial rapid rise in the concentration of the infused amine is followed by a slower rate of increase or a plateauing in the level at 5-10 minutes after the infusion commences (Jones & Blake 1958, Cohen *et al.* 1959, Poole & Watts 1959, Baetz *et al.* 1973). Catecholamine kinetics are linear in that the increments in catecholamine levels are directly proportional to the infusion rate (Jones & Blake 1958, Cohen *et al.* 1959). The half-lives of both noradrenaline and adrenaline in the mammalian bloodstream are very short, variously estimated as less than 10 minutes (Zuspan *et al.* 1965), less than 2 minutes (Baetz *et al.* 1973, Cohen *et al.* 1959, LaBrosse *et al.* 1961), one minute (Miller & Elliot 1954), $\frac{3}{4}$ of a minute (Jones & Blake 1958) and 38 seconds (Filbert & Weller 1959). Little information is available on the kinetics of dopamine in the blood but in sheep it rapidly disappears from the circulation (Kelly *et al.* 1970).

Table 5-2

Plasma Catecholamine Concentrations Found After the Injection or Infusion of Exogenous Catecholamines

Reference	Species	Number	Catecholamine and Form of its Administration	Infusion Rate/ Injection Dose	Preinfusion/ Preinjection Plasma Value (ng ml ⁻¹)	Maximum Plasma Concentration or Mean Plasma Infusion Concentration (±SD) (ng ml ⁻¹)
Miller & Elliot (1954)	dog	8	adrenaline injection	10 µg kg ⁻¹ 5 " 1 "	~7 ~9 ~9	>76 22 12
Jones & Blake (1958)	dog	30	adrenaline infusion	0-5 µg kg ⁻¹ min ⁻¹ (up to 50 minutes)	≤1 —	0-70
Axelrod <i>et al.</i> (1959b)	cat	2	adrenaline infusion	2.5 µg kg ⁻¹ min ⁻¹ (30 minutes)	-	16 and 25
		3	adrenaline injection	70 µg kg ⁻¹		40 (31-47) at 2 minutes post-injection
Cohen <i>et al.</i> (1959)	human	41	noradrenaline infusion	11.6-21.0 µg min ⁻¹	0.6	6
			adrenaline infusion	12.4-21.0 µg min ⁻¹ (up to 65 minutes)	0.2	3

Table 5-2 (continued)

Reference	Species	Number	Catecholamine and Form of its Administration	Infusion Rate/ Injection Dose	Preinfusion/ Preinjection Plasma Value (ng ml ⁻¹)	Maximum Plasma Concentration or Mean Plasma Infusion Concentration (±SD) (ng ml ⁻¹)
Filbert & Weller (1959)	dog	5	adrenaline injection plus infusion	40 µg kg ⁻¹ injection plus 6-7 µg kg ⁻¹ min ⁻¹ (75 minutes)	-	100 (at 30 minutes)
Poole & Watts (1959)	dog	4	adrenaline infusion	1.25 µg kg ⁻¹ min ⁻¹	<1	8.5±2.8
		4		2.50 "	<1	19±32
		5		5.0 "	<1	75±78
		3		10.0 " (37-197 minutes)	<1	201±16 (arterial)
Zuspan <i>et al.</i> (1965)	human	16	adrenaline stepped infusions	0.06 µg kg ⁻¹ min ⁻¹	0.6	2
				0.12 "		2
				0.24 " (20 minutes at each dose)		3
Häggendal & Svedmyr (1966)	human	5	adrenaline infusion	0.1 µg kg ⁻¹ min ⁻¹ (20-30 minutes)	<0.1	0.7
Kral <i>et al.</i> (1969)	human	2	adrenaline infusion	0.1 µg kg ⁻¹ min ⁻¹ (30 minutes)	0	0.5

Table 5-2 (continued)

Reference	Species	Number	Catecholamine and Form of its Administration	Infusion Rate/ Injection Dose	Preinfusion/ Preinjection Plasma Value (ng ml ⁻¹)	Maximum Plasma Concentration or Mean Plasma Infusion Concentration (±SD) (ng ml ⁻¹)
Baetz <i>et al.</i> (1973)	pig	5	noradrenaline infusion	1 µg kg ⁻¹ min ⁻¹ (50 minutes)	0.37±0.09	9.3±13.6
			adrenaline	1 µg kg ⁻¹ min ⁻¹ (50 minutes)	0.09±0.07	5.1±3.6
Christensen <i>et al.</i> (1975)	human	6	adrenaline infusion	6 µg min ⁻¹ (20 minutes)	0.02±0.02	0.71±0.32
Shoulson <i>et al.</i> (1975)	human	1	noradrenaline infusion	1.0-3.0 µg kg ⁻¹ (increasing infusion rate over 75 minutes)	<0.1	~0.1-0.7
Manger <i>et al.</i> (1976)	human	6	noradrenaline infusion	0.05 µg kg ⁻¹ min ⁻¹ (10 minutes)	2.4 (arterial) 3.3 (venous)	3.4 (arterial) 3.6 (venous)
				0.1 µg kg ⁻¹ min ⁻¹ (10 minutes)		4.3 (arterial) 3.9 (venous)

Table 5-2 (continued)

Reference	Species	Number	Catecholamine and Form of its Administration	Infusion Rate/ Injection Dose	Preinfusion/ Preinjection Plasma Value (ng ml ⁻¹)	Maximum Plasma Concentration or Mean Plasma Infusion Concentration (±SD) (ng ml ⁻¹)
				0.2 µg kg ⁻¹ min ⁻¹ (10 minutes)		5.8 (arterial) 5.0
Jones <i>et al.</i> (1977)	sheep	4	adrenaline infusion	0.26 µg kg ⁻¹ min ⁻¹ (2 hours)	<0.05	0.6-8.5 (mean 3.8±2.9)

Conversion of noradrenaline and adrenaline to methoxylated derivatives by catechol-O-methyltransferase appears to be the most important route of metabolic deactivation and is concentrated in the heart, kidney, lung, liver and muscle (Axelrod *et al.* 1959a) but considerable tissue binding and neural uptake also contribute (Whitby *et al.* 1961, Iversen 1975). Tracer studies with a greater sensitivity than available otherwise have demonstrated that the decline of both labelled noradrenaline and adrenaline in plasma are characterized by a very fast initial decay in activity followed by a slow second phase (Axelrod *et al.* 1959b, Whitby *et al.* 1961, Gitlow *et al.* 1964). The fast decay is thought to be the result of distribution, metabolism and uptake into tissues while the persistence of activity in the blood reflects a slow release of the labelled amines from binding sites. In whole mice for example 16% of injected tritiated noradrenaline is still present in an unaltered form in body tissues after 6 hours despite almost total disappearance from the plasma (Whitby *et al.* 1961). Filbert & Weller (1959) estimate the half-lives of the 2 components of the decay curve of adrenaline in the dog as 38 seconds and 23 minutes. In the pig and in man the noradrenaline plasma concentration is higher after a noradrenaline infusion than is the case for adrenaline given a corresponding infusion rate (Cohen *et al.* 1959, Baetz *et al.* 1973). This is reflected in man by a faster rate of removal of tritiated adrenaline from the plasma during the slow phase of its disappearance (half-life of 73.5 minutes) (LaBrosse *et al.* 1961) than tritiated noradrenaline (half-life of 240 minutes) (Gitlow *et al.* 1964). In part the difference arises from the greater influence of adrenaline O-methylation as a mechanism for its removal whereas, in the case of noradrenaline, tissue uptake and storage are more important (Whitby *et al.* 1961).

On the question of endogenous release of catecholamines during catecholamine infusions the literature is limited. Kral *et al.* (1969) found increased noradrenaline levels in human plasma during adrenaline infusions while Robinson & Watts (1962) suggest that canine adrenal adrenaline secretion was suppressed by either

exogenous noradrenaline or adrenaline. This point awaits clarification with a more specific and sensitive assay.

The behavioural effects of peripheral catecholamine administration to humans and animals has received much attention (Lader & Tyrer 1975, Murphy & Redmond 1975). Emotional states of various types, especially agitation and anxiety, have been induced as a result. However in humans the quality of the emotional changes depends not only on the personality of the infused subjects but also on how the external environment is manipulated by the investigators. L-dopa both oral and intravenous is also potent in affecting emotional states but the changes appear complex and have not been systematically studied particularly in healthy individuals (Damasio & Castro Caldas 1975, Murphy & Redmond 1975).

EXPERIMENTAL METHODS

Six 2 year old Dorset Horn ewes, cannulated in pairs, were used in the infusion experiments. The sheep were adapted to the housing and feeding regimes for a minimum of 2 weeks prior to cannulation. After a period of 8 days to permit recovery from the surgery daily catecholamine infusions commenced either until the infusion series had been completed or until cannula failure occurred. The experiment continued over a period of 9 weeks using 3 pairs of cannulated sheep.

Surgery The sheep were starved for 24 hours before surgery and shorn around the neck to permit easy access to the incision site. For surgery the sheep were first lightly anaesthetized with 12-15 ml of sodium pentobarbitone (60 mg ml^{-1}) (Nembutal sodium, Abbot Laboratories, Chicago, Illinois, USA) and maintained under halothane and oxygen (Fluotec 3, Cyprane Ltd, Keighly, UK). The skin over the jugular vein was carefully shaved and the jugular vein exposed through an incision in the neck; the carotid artery was located and cleared from the surrounding connective tissues. The artery was temporarily occluded by applying traction to loose ligatures on either side of a small hole, cut in the vessel with sharp pointed scissors. Each cannula (polyvinyl chloride,

i.d. 1.5 mm, Dural Plastics and Engineering, Dural, NSW, Australia) was passed through and glued through an oblong (20 x 40 mm) of knitted polypropylene mesh (PM-53, Ethicon, Sommerville, New Jersey, USA) 100 mm from the proximal end with polyvinyl-chloride solvent cement (Garnite S'lon, Garnite Plastic Sales Ltd, Auckland, New Zealand) and inserted through the incision in the carotid wall toward the heart until the mesh rested on the vessel surface. It was wrapped around the vessel and the edges sutured together to form a sleeve (Figure 5-1). The same procedure was used to secure a second cannula in the jugular vein. The distal ends of the cannulae were taken under the surface of the skin from the incision site and brought out onto the dorsal surface of the neck. The 2.8 m long cannulae (lumen volume 5.0 ml) were wound onto small reels attached to the wool on the back of the sheep and the ends sealed with blocked and blunted 16 gauge syringe needles. After several hours to permit recovery from the anaesthetic the sheep were transported to the experimental housing facility and together with 2 non-cannulated sheep were placed in metal crates.

Housing Facility and Feeding Regime All sampling, sample processing and infusions were carried out in a separate room by passing the cannulae through a hole in the dividing wall (Figure 5-2). A one-way window enabled the behaviour of the sheep to be observed without disturbance. Hay and water were available *ad libitum* and were replaced with fresh supplies daily at 8 am. At the conclusion of each infusion experiment (usually by 1.30 pm) a small quantity of freshly cut grass was also supplied to each sheep.

Infusions and Blood Sampling At least 2 blood samples were obtained from each sheep on the day of cannulation, one as soon as the cannula was implanted in the carotid artery and another after the sheep had regained consciousness and had been transported to the housing facility. Depending on the time available a variable number of other samples were also collected during and after surgery and the sampling times recorded. All blood

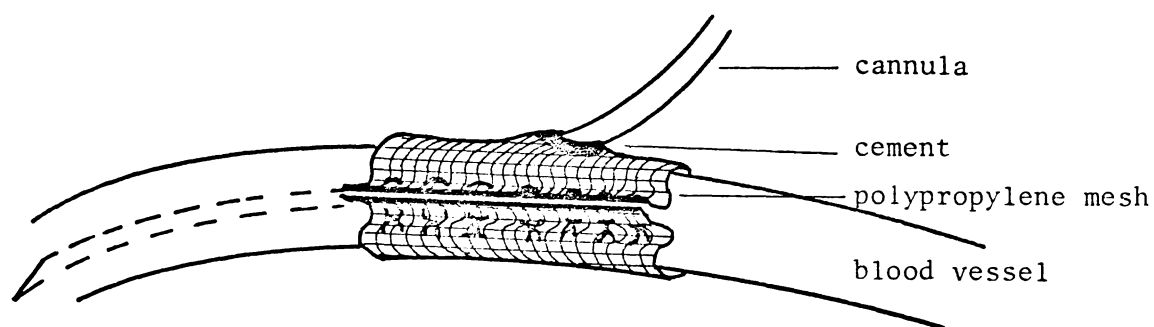


Figure 5-1. Use of polypropylene mesh to secure the cannula to the blood vessel.

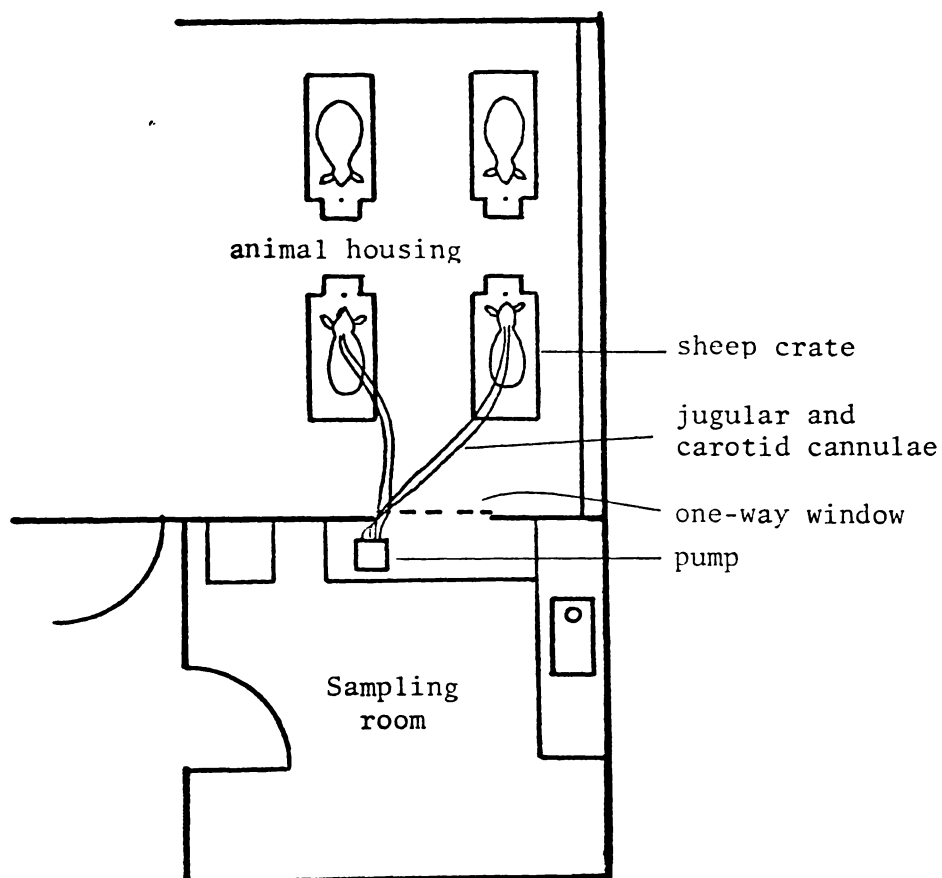


Figure 5-2. Experimental housing and sampling facilities.

samples (7 ml for catecholamine analysis and 4 ml for cortisol analysis) were from the carotid artery while infusions were into the jugular vein using a piston displacement pump (Palmer Super 10 Recording Drum). The faster infusion flow rates (2 ml min^{-1}) necessary to achieve the desired l-dopa doses (because of its limited solubility) necessitated the use of a peristaltic pump (Hiloflow, F.A. Hughes, Epsom, UK). Between experimental infusions a heparinized saline solution (50 IU ml^{-1}) (Heparin Sodium, Commonwealth Serum Laboratories, Melbourne, Australia) was continuously infused at 1.40 ml hr^{-1} through the cannulae to keep them patent. Two carotid artery blood samples were taken daily at approximately 7.30 am and 7.30 pm from the cannulated sheep over the recovery period. The infusion and sampling regimes for each infusion experiment (one per day from 9 am to 1 pm in each cannulated sheep) are shown in Tables 5-3 and 5-4. Blood samples were collected and treated according to the methods in Chapter 4. The total daily blood volume collected from each sheep was approximately 160 ml including a 10 ml sample (preserved with disodium ethylenediamine tetraacetic acid) for haematological tests. The packed cell volume, red blood cell count, white blood cell count and haemoglobin concentrations were determined with a Royco blood cell counter by the staff of the Ruakura Animal Health Laboratory. On the first day of an infusion series blood samples were taken over the course of 4 hours infusing only saline to establish a baseline for subsequent infusions. Catecholamine infusions were approximately 90 minutes in duration after a 30 minute saline infusion to establish baseline levels of the measured substances. At the conclusion of the catecholamine infusion the contents of the venous cannulae were withdrawn and replaced with saline which was infused for a further 2 hours. The catecholamines for infusion were dissolved in saline containing 0.3% ascorbic acid (w/v) as a preservative, filtered through $0.45 \mu\text{m}$ filters (Millipore Filter Corporation, Bedford, Massachusetts, USA) prior to use and stored at 4° . The noradrenaline and adrenaline infusion rates were chosen to approximate a medium and a high rate of endogenous secretion after an examination of the literature on this point. Cannula failure

after a period of days or weeks prevented complete series (all 4 catecholamines at 2 different rates) from being carried out on all but 2 sheep, with partial series obtained from 2 others. Difficulties with cannulae more often related to occlusions at the tip preventing blood withdrawal (but not infusion) rather than the dislodgement of the cannula from the blood vessel or clotting within the lumen.

Table 5-3

Summary of the Catecholamine Infusion Experiments

Sheep Number	Weight (kg)	Cannulation Date	Infusions ($\mu\text{g kg}^{-1} \text{ min}^{-1}$)	
258	56	26. 8.76	-	
254	53	26. 8.76	saline	
			adrenaline	0.33
			adrenaline	1.00
237	44	15. 9.76	saline	
			adrenaline	0.33
			adrenaline	1.00
			noradrenaline	0.33
			noradrenaline	1.00
			dopamine	2.0
			dopamine	6.0
			1-dopa	20.0
			1-dopa	60.0
221	45	15. 9.76	saline	
			dopamine	2.0
			dopamine	6.0
232	47	14.10.76	-	-
205	49	14.10.76	saline	
			adrenaline	0.33
			adrenaline	1.00
			noradrenaline	0.33
			noradrenaline	1.00
			dopamine	2.0
			dopamine	6.0
			1-dopa	20.0
			1-dopa	60.0

Sheep 258 suffered from an infection and despite treatment with antibiotics remained lethargic and in poor appetite over the recovery period. No catecholamine infusions were carried out with this sheep. All other sheep were in good condition during the course of the experiment.

Table 5-4

Catecholamine Infusion and Sampling Regime

Infusion	Time (minutes)	Sampling Times (minutes)	Analyses
saline	-30	-30	
catecholamine	0	0	lactic acid
		15	cortisol
		30	catecholamines
		60	
saline	90	90	
		90+10 sec*	
		+30 " *	
		+60 " *	
		+ 2 min	catecholamines
		+ 5 "	
		+10 "	
		+15 "	
		120	lactic acid
		150	cortisol
stop	210	210	catecholamines

* approximate times only, actual times recorded during each infusion.

Blood Pressure Measurements To investigate the cardiovascular consequences of these catecholamine infusions a second series of experiments was carried out at a later date. Arterial blood pressure changes were measured in sheep during catecholamine infusions at the same rate as in the original series. Four sheep were cannulated, housed and infused with dopamine, nor-adrenaline and adrenaline as described above. Adaptation for 72 hours after cannulation was allowed before the experimental infusions were commenced. Arterial blood pressure was monitored by Statham P23BB pressure transducers (Statham Laboratories, Hato Rey, Puerto Rico) connected through a preamplifier unit to a Sargent-Welch DSRLG 2-channel pen recorder (Sargent-Welch Scientific Company, Skokie, Illinois, USA) situated in the sampling room (Figure 5-2). The pressure transducers were calibrated using a water column and pulse rates were determined from the pulse pressure changes on the recorder. To ensure cannula patency a slow infusion (20 ml hr^{-1}) of heparinized saline 50 IU ml^{-1}) was maintained through the transducer pressure chamber and into the carotid artery using a peristaltic pump (Havard Apparatus, Hillis, Massachussetts, USA). The sheep were infused in pairs; one infusion per day until the series were completed or until cannula failure. The arterial pressure effects of the acute venous administration of propranolol (1 mg kg^{-1}) and phentolamine (1 mg kg^{-1}) were also recorded on separate days (§7.6).

Statistical Analysis An analysis of variance of the cannulation and recovery assay data (logarithmically transformed) was carried out with the following effects examined for significance by the F-test.

1. Differences between individual sheep. (sheep)
2. Differences between the 2 sample groups
(cannulation samples on day 1 compared with
recovery samples on days 2-8). (group)
3. Differences between days within groups. (day w group)
4. Sheep-group interations. (sheep x group)
5. Sheep-days within group interactions. (sheep x day
w group)

RESULTS AND DISCUSSION

Cannulation and Recovery

The analysis of variance of the catecholamine data relating to cannulation (day 1) and recovery (days 2-8) (Table 5-5) show significant differences between individual sheep in both noradrenaline ($p < 0.05$) and adrenaline responses ($p < 0.01$). However the concentrations of noradrenaline in the blood collected while the sheep were under halothane anaesthesia and shortly afterwards (day 1) do not differ significantly from those found in samples collected over the subsequent week, while the difference in the adrenaline levels is significant only at the 10% level (Figure 5-3, Table 5-5).

Table 5-5

Summary of the Analysis of Variance of the Cannulation
and Recovery Catecholamine Data

Effect	Log Noradrenaline	Log Adrenaline
Sheep	$F_{5,45} = 3.23 *$	$F_{5,41} = 4.63 **$
Group ¹	$F_{1,45} < 1 \text{ ns}$	$F_{1,41} = 2.88 +$
Day w group	$F_{6,45} = 2.70 *$	$F_{6,41} = 1.30 \text{ ns}$
Sheep x group	$F_{5,45} = 2.87 *$	$F_{5,41} < 1 \text{ ns}$
Sheep x day w group	$F_{22,45} = 1.23 \text{ ns}$	$F_{22,41} = 1.46 \text{ ns}$

ns = non significant + $p < 0.1$

* $p < 0.05$ ** $p < 0.01$

¹ the data were separated into 2 groups (day 1 and days 2-8)

Overall the blood levels of the catecholamines in the 6 sheep over the cannulation and recovery period are both higher and more variable than the levels recorded during the saline infusions or compared with resting values reported in various mammalian species. There are several possible causes; the strange surroundings, starvation for 24 hours, restraint in administering premedication,

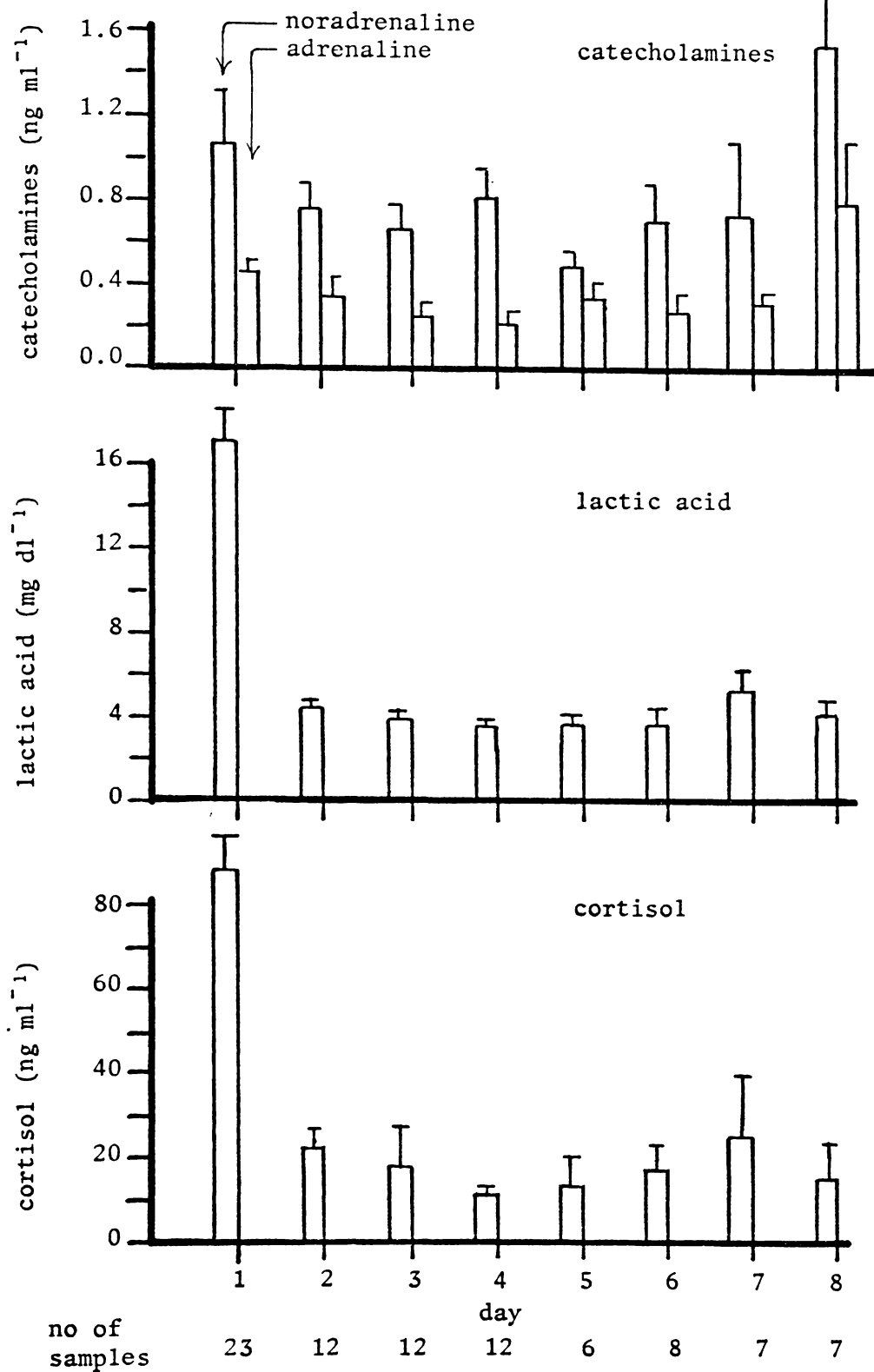


Figure 5-3. Mean ($\pm$ SE) plasma catecholamines, cortisol and lactic acid in 6 sheep on day of cannulation (day 1) and recovery over subsequent 7 days (days 2-8) prior to catecholamine infusions. Over the recovery period samples were collected twice daily from patent cannulae.

and the light anaesthesia would all contribute to an increase in catecholamine levels above baseline during day 1.

Once the sheep were moved to metabolic crates blood samples were taken twice daily prior to feeding. Even though the samples were taken remotely and as quietly as possible the sheep were aware of the presence of the sampler in the next room and the introduction of saline at ambient temperature into the carotid to clear the cannulae before and after sampling may constitute a mild stressor to the sheep. In retrospect saline heated to body temperature would be preferable in assessing basal catecholamine levels.

Towards the end of the recovery period there was an increase in plasma catecholamines (especially marked on day 8) and in the case of noradrenaline a significant difference ($p < 0.05$) emerged between catecholamine levels within days 2-8. It is possible that an association was formed by the sheep in a Pavlovian manner between sampling and feeding over this period. Anticipation of feeding has been found to cause increments in bovine plasma corticosteroids (Willett & Erb 1972). Holley *et al.* (1975) attributed one of 2 circadian cortisol peaks in sheep to anticipation of the daily feeding session.

Catecholamine Infusions

Haematological Results The effect of the daily blood sampling regime was to produce a slow but steady decline in the packed cell volume paralleled by a decline in the haemoglobin concentration (Table 5-6).

In the 2 sheep which underwent the full infusion series (205 and 237) the fall in the packed cell volume and the haemoglobin concentration was of the order of 20%. White and red blood cell counts fluctuated from day to day but showed no overall consistent trends.

Table 5-6

Haematological Results from the Sheep Before and After
the Catecholamine Infusion Series

		Sheep 205		Sheep 221		Sheep 237	
		Before	After	Before	After	Before	After
PCV	(%)	25	20	24	22	28	23
Hgb	(g dl ⁻¹)	9.6	7.4	9.7	8.2	8.6	6.8
RBC	(x10 ¹² l ⁻¹)	5.57	5.80	6.56	6.10	5.69	5.34
WBC	(x10 ¹² l ⁻¹)	10.3	8.6	11.0	13.4	11.3	12.6

PCV packed cell volume

Hgb haemoglobin concentration

RBC red blood cell count

WBC white blood cell count

Behaviour Detailed records of the behaviour of both infused and non-infused sheep were made during these experiments but the results are not included here for reasons of space and relevance. However it can be noted that the onset of noradrenaline, and particularly adrenaline, infusion, was sometimes marked by agitated behaviour ranging from restlessness, shaking, and foot stamping to an attempt to jump from the crate in an extreme case. This agitated behavioural phase generally lasted only a few minutes but all catecholamine infusions were characterized by a reduction in food intake and cudding compared to non-infused sheep. A further period of restlessness of short duration often occurred on termination of the catecholamine infusion.

Plasma Catecholamines Saline infusions had no significant effects on catecholamine levels in the plasma although sheep 237 had higher and more variable noradrenaline and adrenaline concentrations than other subjects (Table 5-7).

Table 5-7

Mean Plasma Catecholamine Concentrations ($\pm$ SD)
in Saline-infused Sheep

Sheep	Noradrenaline (ng ml ⁻¹)	Adrenaline (ng ml ⁻¹)
205	0.36 $\pm$ 0.18	0.19 $\pm$ 0.18
221	0.29 $\pm$ 0.09	0.36 $\pm$ 0.64
237	0.81 $\pm$ 0.50	0.41 $\pm$ 0.45
254	0.07 $\pm$ 0.08	0.20 $\pm$ 0.10

Typical individual plasma response curves during dopamine, noradrenaline and adrenaline infusions at the two different rates are presented in Figure 5-4. L-dopa kinetics fall into a separate class and are treated separately (§5.3). Catecholamine infusions were associated with a rapid rise in hormone level followed by a slower rise to reach a maximum plasma concentration at the arrest of the infusion. The maximum levels achieved by the infused catecholamines at the two different infusion rates indicate an approximately linear response. On infusion termination a sharp decline occurred in the catecholamine concentration. To this point then there is general agreement with the preceding studies (Table 5-2).

However the fall in catecholamine levels from the peak infusion value was not always uninterrupted. Rather, considerable adrenergic activity occurred in some sheep particularly associated with the higher infusion rates of noradrenaline and adrenaline. The activity was expressed as peaks of endogenous catecholamines (confined to the first 20 minutes postinfusion) sometimes of greater height than levels seen during the infusion. The plasma catecholamine levels immediately before and after the termination of all infusion series are plotted separately in Figure 5-5 on an expanded time scale. Dopamine administration was followed by a period of fluctuating and at times relatively high levels of

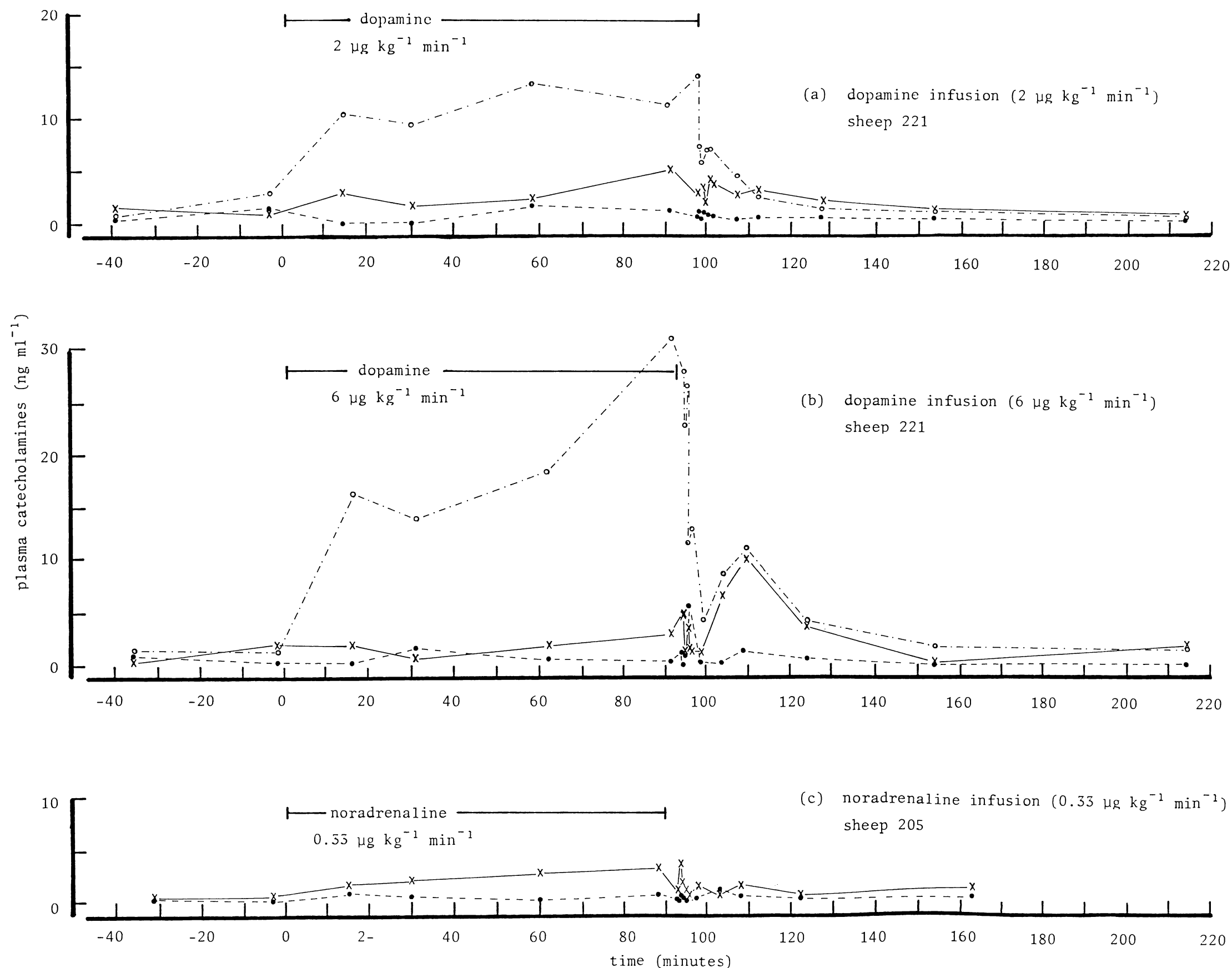


Figure 5-4 (a)-(c). Plasma catecholamine concentrations in individual sheep infused intravenously with dopamine and noradrenaline for 90 minutes from $t=0$.

o---o dopamine x—x noradrenaline •---• adrenaline

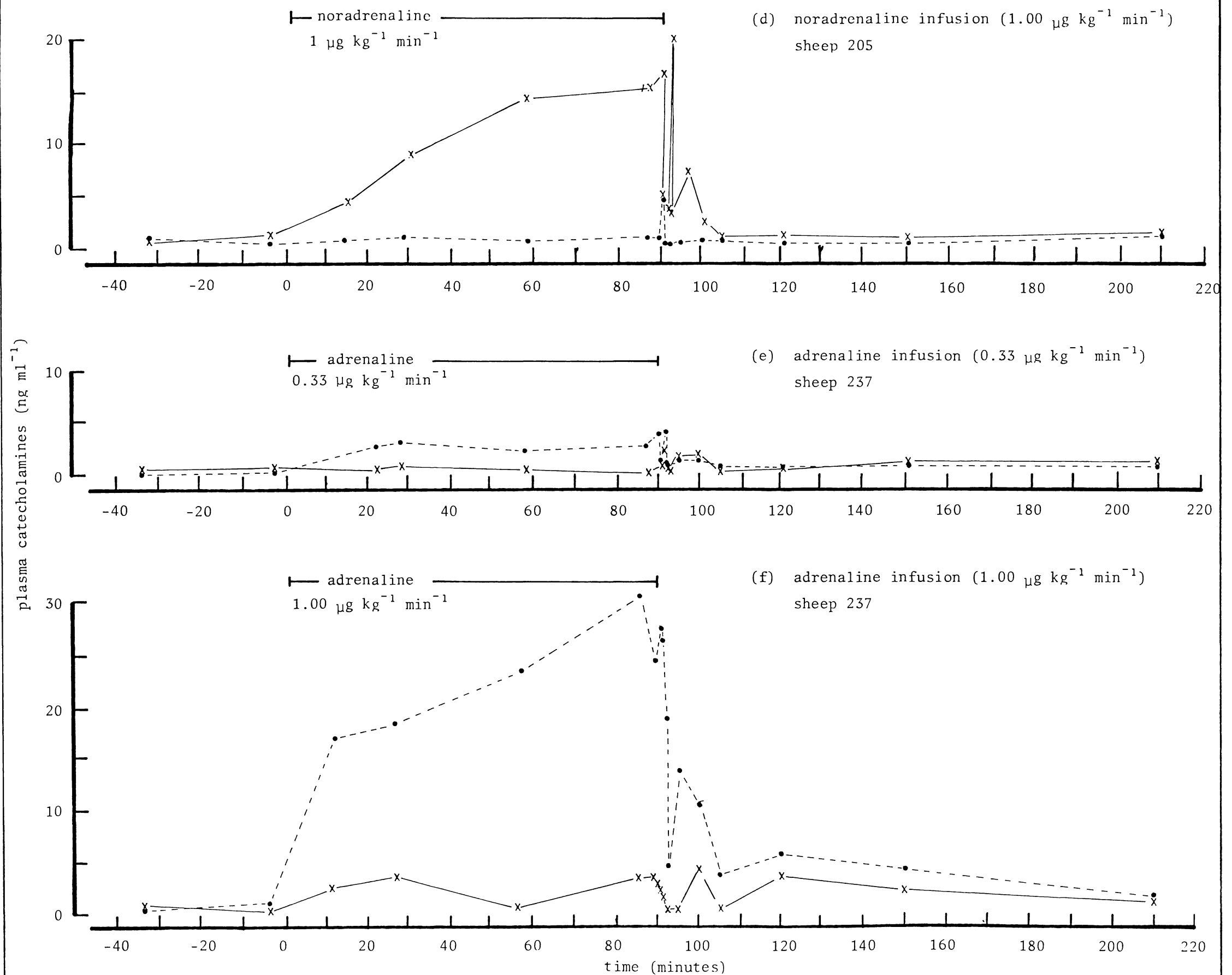
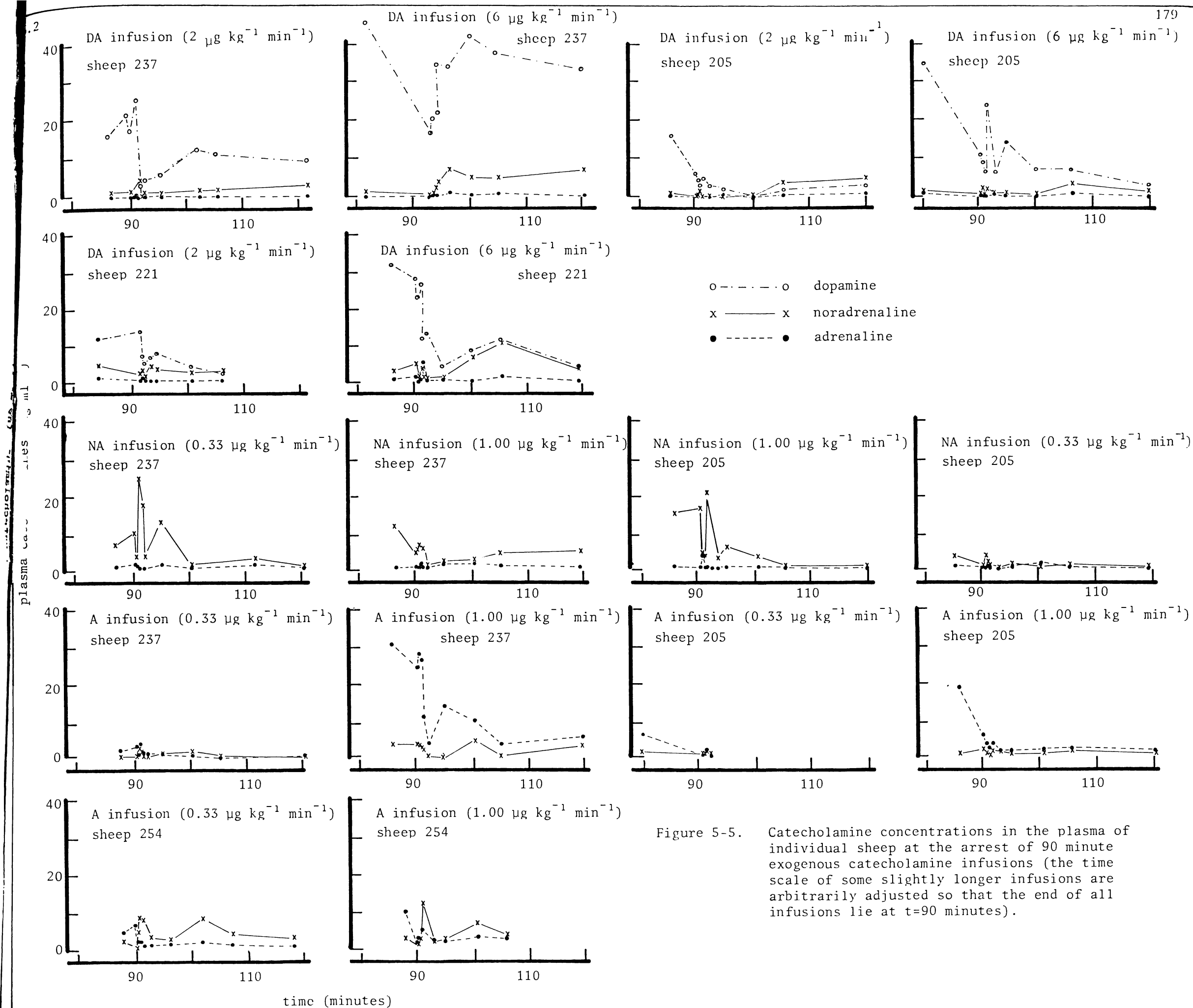


Figure 5-4 (d)-(f). Plasma catecholamine concentrations in individual sheep infused intravenously with noradrenaline and adrenaline for 90 minutes from $t=0$.

x — x noradrenaline

• - - • adrenaline



plasma dopamine and to some extent of noradrenaline also (Figure 5-4 (a),(b)). Noradrenaline infusions seemed to invoke the most marked activity of blood catecholamines post-infusion with some very high noradrenaline peaks observed sometimes accompanied by adrenaline (Figure 5-4 (c),(d)). A similar response was seen on termination of adrenaline infusions usually with rises in noradrenaline as well (Figure 5-4 (e), (f)). Several of the non-infused catecholamine responses have multiphasic dynamics with high release rates immediately on infusion termination and again at about 10 minutes postinfusion (Figures 5-4 5-5). The release of these endogenous catecholamines again varied greatly between sheep from no response to very high peaks.

The transient peaks were an unexpected observation and make an accurate estimation of the half-lives difficult, except to say that they are short and probably less than one minute in the sheep. Any explanation of these observations must explain the endogenous catecholamine secretion observed, its timing and relationship to the infusions, as well as the suggestion that the infusion of any particular catecholamine leads to a subsequent release of the same catecholamine. The following hypothesis is advanced to account for the adrenergic activity seen in sheep on the termination of catecholamine infusions.

1. During the infusion the catecholamine is partly deactivated by sequestration (uptake) into the sympathetic nervous system (§2.4).
2. The arterial blood pressure rises at the start of the infusion in response to these amines and drops on infusion termination possibly resulting in the development of temporary hypotension.
3. The rapid decline in blood pressure and/or the development of hypotension causes a reflex discharge of the sympathetic nervous system liberating stored catecholamines.

4. The released catecholamines are a mixture of endogenous noradrenaline and adrenaline from sympathetic nerves and the adrenal medullae but also include exogenous infused catecholamines gathered by these structures during the preceding infusion.

This theory is based on the following experimental evidence.

Uptake of Exogenous Circulating Catecholamines The mechanism of neural uptake of released catecholamines (§2.4) and its importance to the inactivation of circulating catecholamines have already been considered. Exogenous dopamine, noradrenaline and adrenaline are known to be taken up by the heart for example both *in vivo* and *in vitro* (Raab & Gigg 1955, Iversen 1963, Hellmann *et al.* 1971) and so may become available for subsequent release. Hedqvist & Stjärne (1969) concluded that reuptake and reuse of noradrenaline are major mechanisms in the maintenance of transmitter output during prolonged nerve stimulation, and that noradrenaline synthesis is only necessary to cover the small losses to the circulation and catabolism. The pressor effects of indirect sympathomimetics such as tyramine and nicotine (which act in part by releasing catecholamines from neural stores) or of direct nerve stimulation are potentiated by prior catecholamine infusions; a phenomenon believed to arise from the increased transmitter stores available (Gillespie & Muir 1967b). Somewhat similar mechanisms, though of less physiological significance, occur with respect to l-dopa and dopamine. L-dopa administration results in appreciable dopamine accumulation not only in the brain (§8.2) but also in peripheral tissues such as in the rat heart (combined with a reduction in levels of noradrenaline) (Louis *et al.* 1974). Dopamine has been shown to be taken up and retained in the same particulate cellular fraction as noradrenaline though compared with noradrenaline the uptake and granule sequestration are less marked (Musacchio *et al.* 1966, Collins & West 1968). In view of its comparatively weak sympathetic effects dopamine may also act as a "false transmitter" resulting in diminished physiological responses to pre- and postganglionic

sympathetic nerve stimulation (Farmer 1965, Liu *et al.* 1971) though its rapid intraneural conversion to noradrenaline (Hellmann *et al.* 1971) and metabolic degradation diminish such sympathetic attenuation (Hornykiewicz 1971).

Cardiovascular Effects of Prolonged Catecholamine Infusions

The cardiac and vascular actions of the acute administration of noradrenaline and adrenaline are well known (Neil 1975) and need no further amplification. In man dopamine increased myocardial contractility and cardiac output by a direct action and also indirectly by releasing endogenous noradrenaline (Goldberg 1972). High doses of dopamine may have a vasoconstrictor effect due to α -adrenergic stimulation associated with an increase in arterial blood pressure and peripheral resistance (Rubenson 1971). In smaller doses and in some species β agonist-like properties are seen in renal and mesenteric vasodilation and the decrease in peripheral resistance and blood pressure (Goldberg 1972, Morishita & Furukawa 1974). The cardiovascular and renal effects of orally administered l-dopa are similar to those produced by intravenous dopamine (presumably since l-dopa is converted to dopamine peripherally (§5.4)) except that l-dopa may also exert a central hypotensive effect. Hypotension is a common side effect seen in patients with Parkinson's disease treated with l-dopa (Goldberg 1972, Bianchine & Shaw 1976). In some circumstances l-dopa may induce short-term pressor responses mediated by its conversion to noradrenaline (Eden & Nasmyth 1974).

Blood pressure changes in noradrenaline and adrenaline infusions are characterized by an initial sharp rise in the arterial pressure, a subsequent slow decline during the infusion and a precipitous fall, often into hypotension, at the termination of the infusion (Freeman *et al.* 1941, Lewis & Nickerson 1942, Gilbert & Hohf 1964, Moss *et al.* 1966). The response described is common to all examined mammalian species including sheep (Halmagyi *et al.* 1967). If the infusion rate is sufficiently high hypotension may develop during the infusion and profound circulatory failure and death may ensue (Lewis & Nickerson 1942, Gilbert & Hohf 1964). The severity and duration of "catecholamine shock" depends on the

dose rate and infusion time, subject to considerable individual variation (Gilbert & Hohf 1964). The condition resembles in some respects haemorrhagic shock (Halmagyi *et al.* 1967) and is sometimes seen in phaeochromocytoma after secretory episodes as well as following tumour removal (Engel *et al.* 1942). Post mortem examination of animals after such catecholamine infusions has revealed diffuse myocardial destruction and haemorrhagic lesions affecting particularly the heart but also the kidneys, liver and adrenals (Freeman *et al.* 1941, Lewis & Nickerson 1942, Gilbert & Hohf 1964, Schenk & Moss 1966).

Cardiovascular performance is augmented during the initial phase of catecholamine infusions resulting in an initial peak in blood pressure followed by a dose-related decline in cardiac output, systemic pressure and left ventricular work and an increase in peripheral vascular resistance (Moss *et al.* 1966). The genesis of the initial blood pressure peak has been ascribed to β -adren-ergic cardiac stimulation and an increase in aortic blood flow - since it is suppressed by propranolol (1 mg kg^{-1}) but not by phenoxybenzamine (1 mg kg^{-1}) (Caldwell & Heiffer 1973).

The reasons for the cardiovascular responses becoming increasingly refractory to the pressor effects of infused catecholamines have not been satisfactorily explained. One older hypothesis is that a reduced plasma volume is caused by vasoconstriction which, if of sufficient intensity and duration, leads to anoxia of the peripheral tissues, an increase in capillary permeability and a loss of plasma from the bloodstream into the tissues (Freeman *et al.* 1941). It has also been suggested that the effect is due to a decrease in peripheral resistance caused by desensitization or tachyphylaxis of the adrenergic receptors (Rosenthale & Dipalma 1963) or the vasodilator actions of the infused sympatho-mimetic itself (Mazurkiewicz 1968). A decreased cardiac output resulting from the myocardial damage has also been advocated (Moss *et al.* 1966, Gillespie & Muir 1967a). Acidosis associated with catecholamine infusions may be partly instrumental in the progressive reduction in the cardiovascular responses (Atkinson

et al. 1972). However it is true that all these suggestions have been discounted at other times as a cause of the loss in pressor response to catecholamines.

Infusion rates ending in hypotension and shock are generally higher than used in the present study. For example 3.4-16.4 $\mu\text{g kg}^{-1} \text{ min}^{-1}$ of adrenaline for up to 2 hours (Freeman *et al.* 1941) and 2-18 $\mu\text{g kg}^{-1} \text{ min}^{-1}$ of noradrenaline for 2 hours (Gilbert & Hohf 1964) induce shock in dogs. The study of sheep by Halmagyi (1967) involved adrenaline infusion rates of 0.65-2.53 $\mu\text{g kg}^{-1} \text{ min}^{-1}$ over 3 hours. Cardiac output declined in these animals during the infusion. After withdrawal of the infusion both cardiac output and systemic arterial pressure fell below control levels. The α receptor blocking agent phenoxybenzamine prevented the rise in arterial pressure but not the fall below control after discontinuing adrenaline. Combined α and β blockage during infusion saw only a moderate arterial pressure rise but development of severe hypotension 4-5 hours after the infusion resulting in death. Neither hypovolaemia nor heart failure was found to be responsible for the postadrenaline hypotension in sheep.

Catecholamine Release in Haemorrhage and Hypotension Hypotension as a result of haemorrhage has long been known to be a potent stimulus to catecholamine secretion (Callingham 1975), mediated through reflex control via carotid sinus receptors (Kaindl & von Euler 1951). A drop in blood volume and pressure in haemorrhage produces a rapid increase in the output of noradrenaline and adrenaline proportional to the blood loss (Zileli *et al.* 1974). The mean output of noradrenaline and adrenaline in the right lumbo-adrenal vein in dogs subject to a $\frac{1}{3}$ blood volume depletion increased from 2.3 ± 1.2 and $5.4 \pm 2.0 \text{ ng kg}^{-1} \text{ min}^{-1}$ (n=15) to a maximum at 60 minutes of 58.0 ± 70.5 and $250.0 \pm 162.7 \text{ ng kg}^{-1} \text{ min}^{-1}$ (n=5) (Zileli *et al.* 1974). The rise in adrenaline was especially pronounced with haemorrhage greater than 40 ml kg^{-1} and an arterial pressure lower than 60 mm Hg. It should be

noted that an output of this magnitude is of the same order of magnitude as the infusion rates employed in the present experiment.

Blood Pressure Experiments

While the cardiovascular adaptations occurring during and after catecholamine infusions seem a plausible mechanism to account for the catecholamine response curves, the infusion rates associated with postinfusion hypotension discussed in the literature have been higher than in the present study. Furthermore there are few data relating to the sheep. Therefore a second experiment was undertaken to investigate the effects of dopamine, noradrenaline and adrenaline infusions (at the same rate and over the same interval as previously) on arterial blood pressure.

The overall results (Figure 5-6) show that at these dose levels the cardiovascular effects of catecholamine infusions described in other species also occur in sheep, though postinfusion hypotension did not always occur. The effect was increasingly marked from the lowest level of dopamine through to the highest level of adrenaline. Dopamine had little effect on either heart rate or blood pressure, though a slight pressor effect was sometimes discernible (Figure 5-6). Noradrenaline, at the higher infusion rate in particular, caused initial hypertension followed by hypotension as well as accelerating the heart rate (Figure 5-7). Adrenaline possessed the most potent cardiovascular effects with large perturbations to both measured parameters (Figure 5-8). The heart rate was increased and tended to remain high for considerable periods following the adrenaline infusion. Marked cardiac arrhythmia was an occasional result of both noradrenaline and adrenaline infusions. An interesting feature of some blood pressure responses to adrenaline and, to a lesser degree, noradrenaline was the occurrence of transient pressure peaks during the rapid fall in blood pressure on infusion termination and during the hypotensive phase. The interesting possibility arises that these oscillations may correspond to

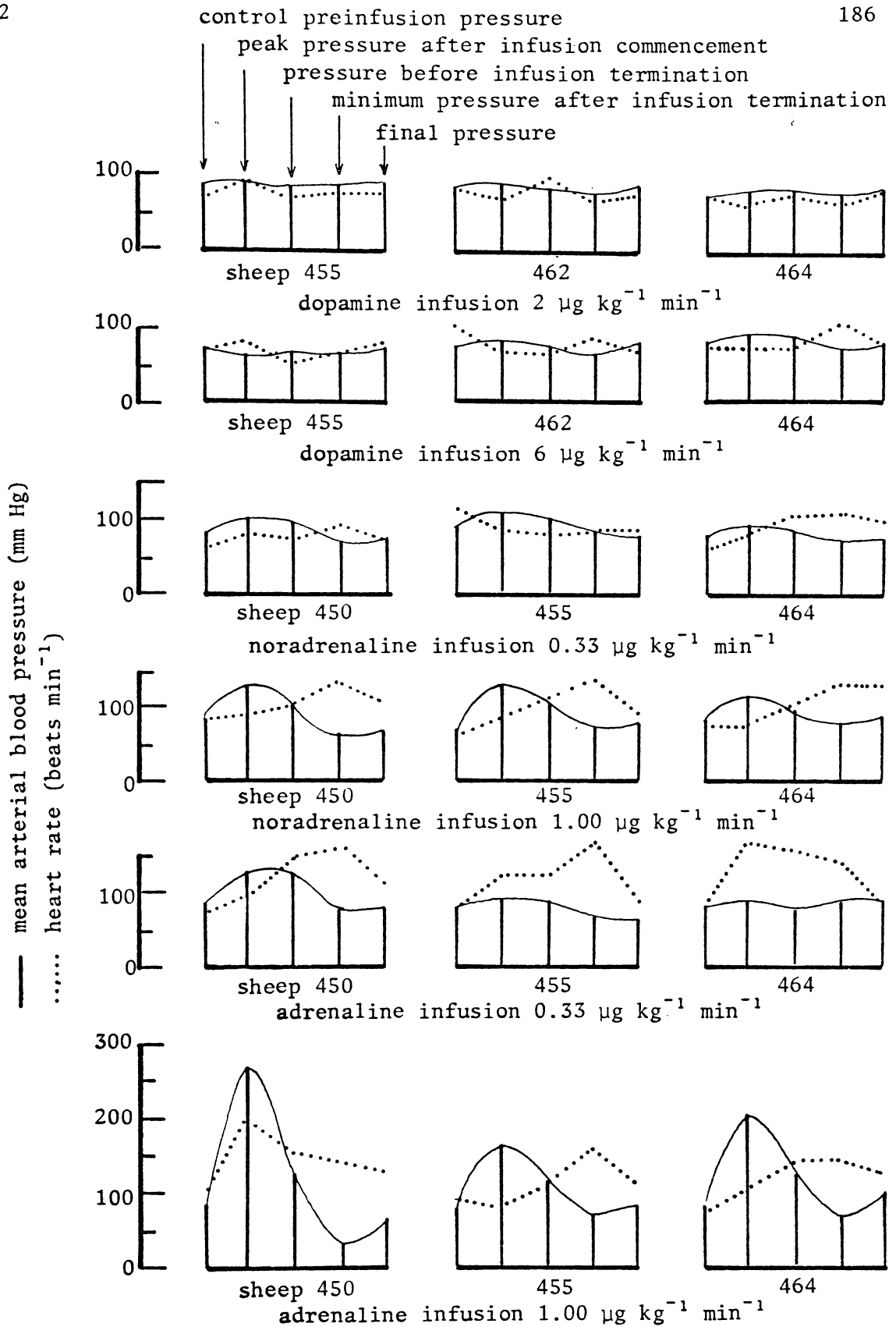


Figure 5-6. Mean arterial blood pressure (with concurrent heart rate) of individual sheep at 5 separate points before, during and after 90 minute catecholamine infusions ((1) preinfusion pressure (2) peak pressure after catecholamine infusion commencement (3) pressure immediately before infusion termination (4) minimum pressure after infusion termination (5) final pressure 2 hours after infusion termination).

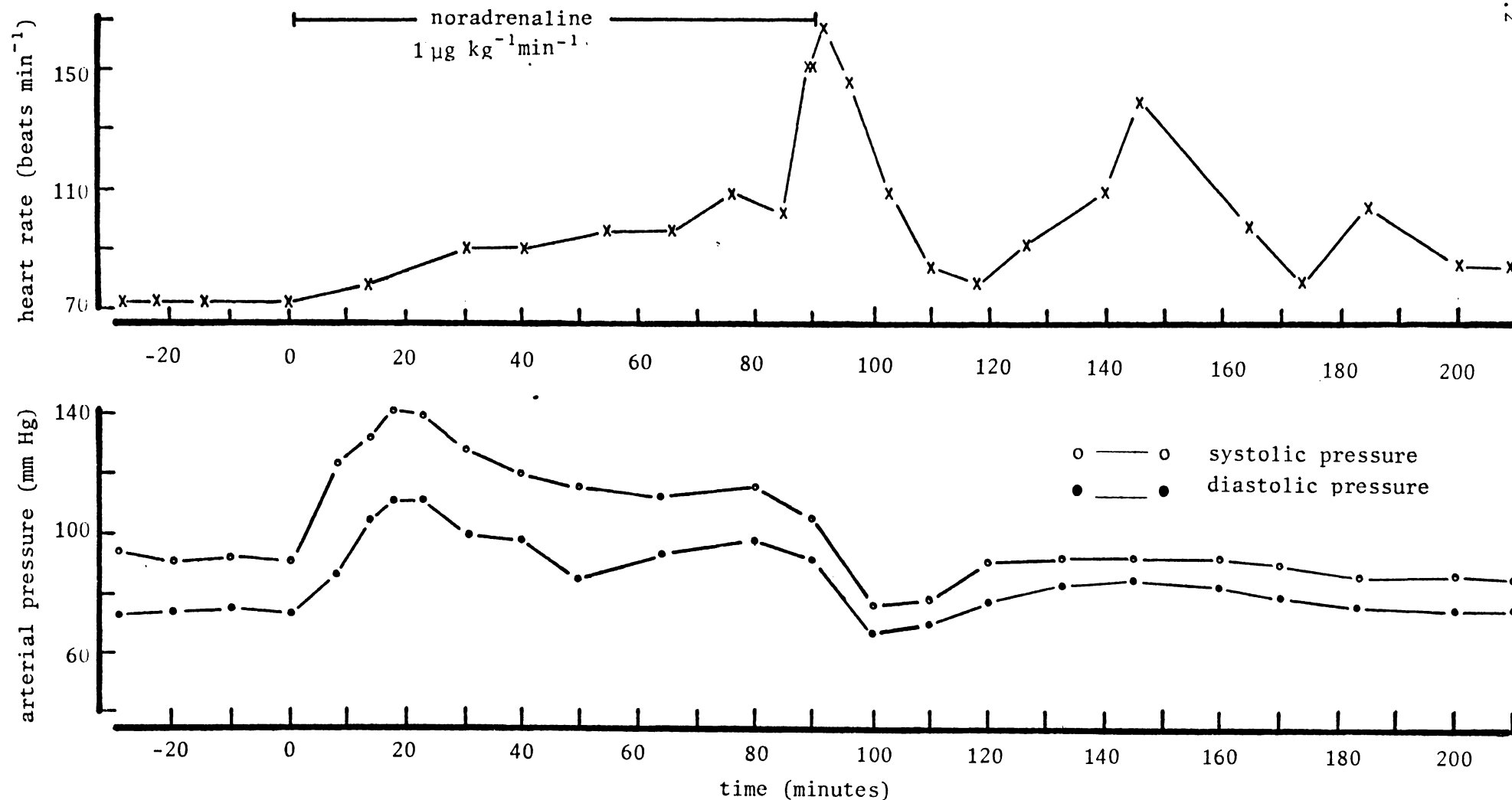


Figure 5-7. Effect of a 90 minute noradrenaline infusion ($1 \mu\text{g kg}^{-1} \text{min}^{-1}$) on the arterial blood pressure and heart rate of sheep 455.

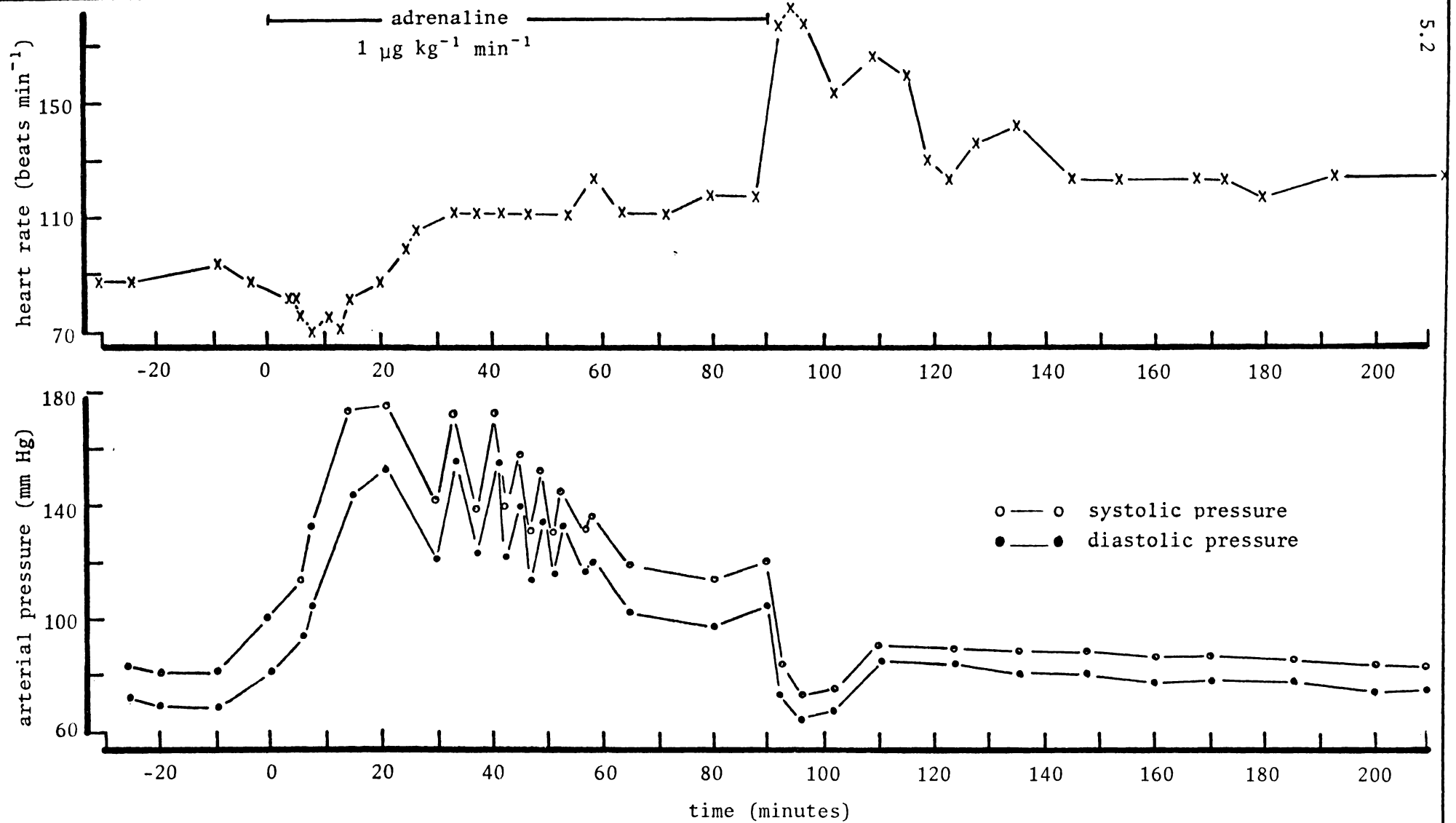


Figure 5-8. Effect of a 90 minute adrenaline infusion ($1 \mu\text{g kg}^{-1} \text{min}^{-1}$) on the arterial blood pressure and heart rate of sheep 455.

surges in circulating catecholamines such as those measured (Figures 5-4, 5-5).

The blood pressure responses observed therefore suggest that the plasma profiles of adrenaline and noradrenaline in these infusions can be explained in terms of the cardiovascular consequences. The phenomenon has not been reported in other species since sampling has not been carried out sufficiently rapidly or over a sufficient period of time following the infusion arrest. In addition if the hypothesis developed here is tenable then adrenergic responses of this type would not be expected to follow low infusion rates, infusions of short duration or tracer infusions. However Filbert & Weller (1959) speculated that the slow rate of disappearance of adrenaline seen in dogs 5 minutes and longer after adrenaline infusions was possibly due either to the release of tissue-bound amines or to sympathetic discharge associated with low blood pressures developed by the procedure.

The lack of a marked effect of dopamine on cardiovascular parameters at the dose levels employed requires further study of the comparatively high level of circulating catecholamines seen sometimes after dopamine intravenous infusion. It may be that the systemic pressure homeostatic system is more sensitive than the pressure transducer used in the present experiment. Furthermore if dopamine was acting as a false neurotransmitter (Kopin 1968) then its comparative lack of cardiovascular potency might also be expected to result in continued reflex stimulation of the nervous system to a cardiovascular demand.

5.3 PHARMACOKINETICS OF L-DOPA IN SHEEP

INTRODUCTION

The behaviour of exogenously introduced l-dopa in the plasma of sheep differs somewhat from the other catecholamines (§5.2). L-dopa is not a normal plasma constituent since its conversion to dopamine occurs rapidly and it is not present in the catecholamine release pool of nervous structures (§2.2). Nor does

l-dopa appear to be taken up into neural tissue with the same affinity as shown for other catecholamines (Iversen 1975) or metabolized quite as rapidly.

Much work has been devoted to the investigation of the plasma pharmacokinetics of l-dopa since the advent of the clinical use of this substance in the treatment of Parkinson's disease (§8.2). Large doses of l-dopa are administered orally to patients with Parkinson's disease in an attempt to saturate enzymes active in the peripheral metabolism of this compound. Even at its peak concentration the plasma content of l-dopa may represent less than 1% of the total oral dose (Tyce *et al.* 1970). The principal metabolic products of oral l-dopa are 3-O-methyldopa, dopamine, 3-methoxy-4-hydroxyphenylacetic acid and 3,4-dihydroxyphenylacetic acid (Hinterberger 1971, Louis *et al.* 1974, Rivera-Calimlin 1974). Of these, the latter two metabolites (both derived from dopamine) greatly predominate. The enzyme which catalyzes the conversion of l-dopa to dopamine, dopa decarboxylase, is widely distributed throughout the body (§2.2). Granerus *et al.* (1973) have shown that orally administered l-dopa was decarboxylated to the extent of $73.8 \pm 6.0\%$ in the gastrointestinal organs of 11 patients with Parkinsonism. The liver by comparison appears to play a minor role, although the evidence here is somewhat in conflict (Mearrick *et al.* 1975). Treatment with l-dopa in man and rats results in only small increases in the urine content of noradrenaline and adrenaline compared to that of dopamine (1240 times control levels in patients on an average daily dose of 4.2 g of l-dopa) (Louis *et al.* 1974). But even this increase in dopamine excretion accounted for only about 6% of the administered dose. L-dopa administration also results in an accumulation of dopamine in the tissues associated with a reduction in the levels of noradrenaline (Louis *et al.* 1974); an observation consistent with the phenomenon discussed in the preceding section.

Blood cells appear to have a significant capacity to take up l-dopa and erythrocyte metabolic conversion of l-dopa to dopamine (but not to 3-methoxy-4-hydroxyphenylacetic acid or 3,4-dihydroxyphenylacetic acid) also occurs slowly (Hinterberger 1971, Mearrick *et al.* 1975). When l-dopa was added to whole blood to a final concentration of $5 \mu\text{g ml}^{-1}$ the packed cell l-dopa concentration rose to 390 ng ml^{-1} in 3 hours of which 80% was subsequently released into l-dopa-free plasma over one hour (Hinterberger 1971). Five to ten percent of plasma l-dopa appears to be protein bound (Hinterberger 1971).

Very little work has been published relating to the kinetics of l-dopa after its direct introduction into the bloodstream. The data available on the plasma responses to oral l-dopa are not applicable to the present results in view of the absorption delay and the buffering action of the gut pool. A half-life of 1.0-1.6 hours in the rat after oral dosing has been reported (Cheng & Fung 1976) while that of injected l-dopa in humans is 55 ± 4.8 minutes ($n=11$) (Granerus *et al.* 1973).

EXPERIMENTAL METHODS - See §5.2

RESULTS AND DISCUSSION

Four l-dopa plasma response curves were obtained from 2 sheep infused at 2 different rates ($20 \mu\text{g kg}^{-1} \text{ min}^{-1}$ and $60 \mu\text{g kg}^{-1} \text{ min}^{-1}$). In the first 90 seconds following infusion termination all 4 curves show a rapid drop in l-dopa concentration followed by a small partial recovery and then a continuous fall over the next 2 hours (Figure 5-9a). It is difficult to dismiss completely the pause in the initial fall as an assay artefact since it occurred on all 4 occasions. One possibility is that it may relate to cardiovascular changes (Eden & Nasmyth 1974) leading to an alteration in the plasma packed cell volume ratio or to a rapid redistribution of l-dopa between the plasma and the blood cells or some other tissue. When the peak plasma concentration of l-dopa, reached at the end of the infusion, is set equal to

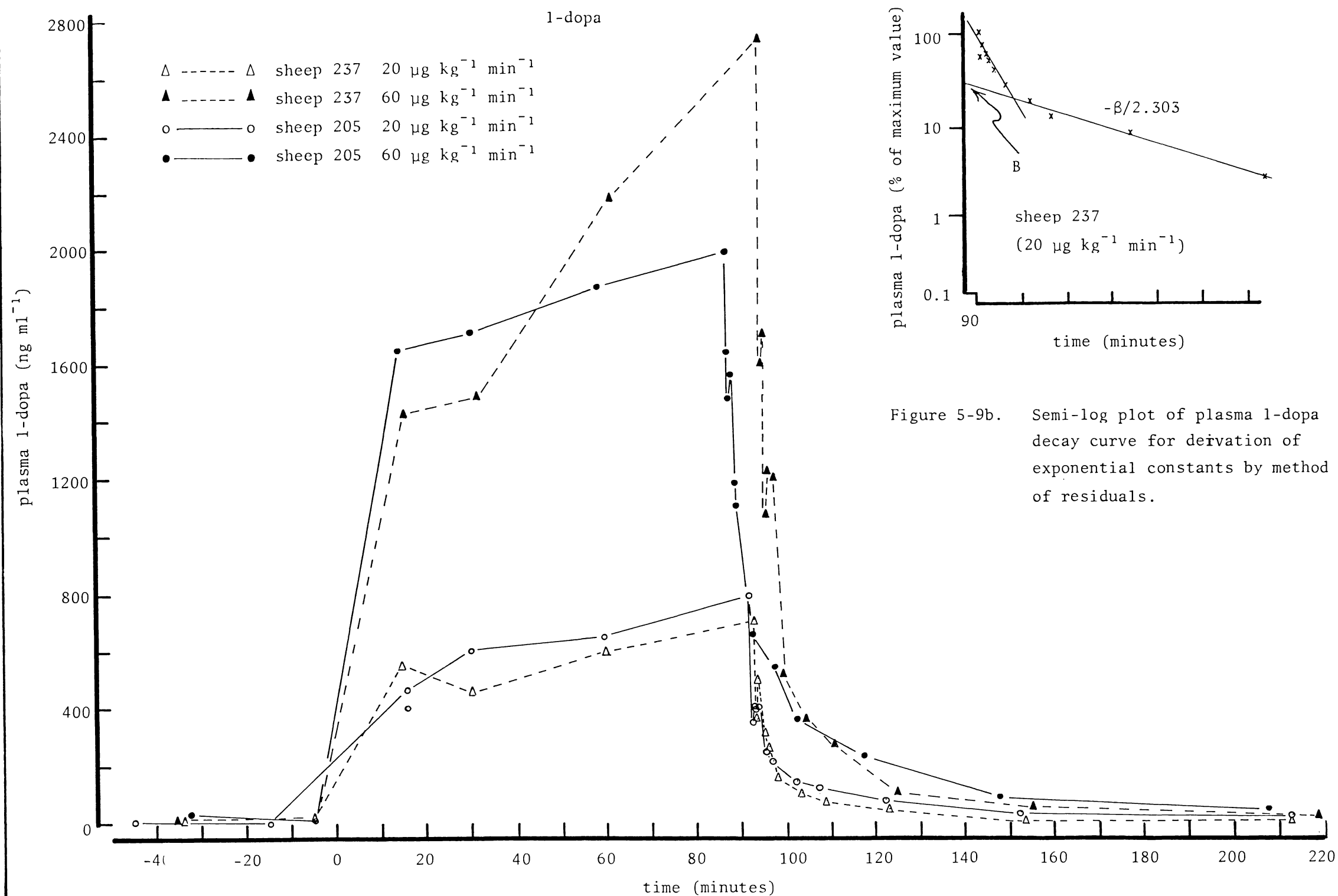


Figure 5-9a. L-dopa plasma response curves in two sheep infused with l-dopa at 20 and $60 \mu\text{g kg}^{-1} \text{min}^{-1}$ for 90 minutes.

Figure 5-9b. Semi-log plot of plasma l-dopa decay curve for derivation of exponential constants by method of residuals.

100.0 and all other concentrations are scaled accordingly the shapes of the 4 response curves are similar (Figure 5-11).

Exponential series of the form

$$C = Ae^{-\alpha t} + Be^{-\beta t} + \dots \quad (1)$$

[where C is the plasma concentration; α , β , A, B are constants; t is the time]

are frequently used to describe first-order drug kinetics. The exponential series may be described graphically by a semi-log plot of drug concentration in plasma versus the time. The linear portions of the curve represent the individual exponential terms and have slopes of $-\alpha/2.303$ and $-\beta/2.303$ for a 2 term exponential while the extrapolated zero time intercepts (after resolving the curve into its components by the method of residuals (Gibaldi & Perrier 1975)) yield A and B. It was apparent after plotting the present results that 2 and perhaps 3 exponential terms are necessary to describe the l-dopa pharmacokinetics in sheep (Figure 5-9b). With only 2 points however the 3rd exponential is ill-defined. The approximate coefficients and exponentials were determined by the method of residuals and entered as initial estimates in the computer programme SAAM (Simulation, Analysis, And, Modelling) (Berman & Weiss 1974) for a least squares fitting of the data. Fitting to both 2 and 3 term exponentials was attempted. The data fit to a 2-term exponential was significantly better than could be made with a 3-term exponential. A 2-term exponential representation was therefore adopted and the final estimated parameters are shown in Table 5-8.

The mean values ($\pm$ SD) of the estimates

α	0.405 $\pm$ 0.032	min ⁻¹
β	0.0305 $\pm$ 0.00392	min ⁻¹
A	58.4 $\pm$ 7.9	
B	22.4 $\pm$ 7.1	

for the 4 constants are used in the remainder of this discussion. The half-lives of the 2 exponential components may now be derived.

Table 5-8

SAAM Estimates of Exponential Constants

	Sheep 237		Sheep 205	
	20 µg kg ⁻¹ min ⁻¹	60 µg kg ⁻¹ min ⁻¹	20 µg kg ⁻¹ min ⁻¹	60 µg kg ⁻¹ min ⁻¹
α	0.448 (17.0)*	0.374 (15.3)	0.387 (23.0)	0.410 (17.5)
β	0.0339 (7.3)	0.0323 (10.8)	0.0250 (11.6)	0.0305 (7.7)
A	60.3 (9.8)	64.5 (9.6)	46.9 (12.0)	62.3 (8.1)
B	20.4 (9.7)	14.3 (18.4)	23.5 (10.7)	31.3 (8.9)

* % fractional standard deviations estimated from the data fitting.

[data scaled to % of maximum value]

$$t_{1/2}^1 = \frac{0.693}{\alpha} = 1.71 \text{ min} \tag{2}$$

$$t_{1/2}^2 = \frac{0.693}{\beta} = 22.72 \text{ min} \tag{3}$$

A biexponential decline in the plasma levels of any compound may be represented by a 2-compartmental model of which there are a number of different (but mathematically indistinguishable) arrangements (Gibaldi & Perrier 1975). Most commonly, in the absence of evidence to the contrary, the model in Figure 5-10 is selected.

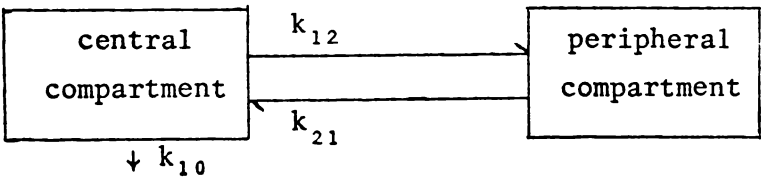


Figure 5-10. 2 compartment model representing 1-dopa pharmacokinetics in sheep

(where k_{12} and k_{21} are the apparent first order intercompartmental distribution rate constants and k_{10} the apparent first order elimination rate constant from the central compartment.)

The rate of change in the amount of substance in the central compartment is described by the differential equation

$$\frac{dX_c}{dt} = k_{21} X_p - k_{12} X_c - k_{10} X_c \quad (4)$$

where X_c and X_p are the amounts of substance in the central and peripheral compartments respectively.

Furthermore it may be shown that the rate constants are related to A, B, α and β by the following relationships

$$\alpha + \beta = k_{12} + k_{21} + k_{10} \quad (5)$$

$$\alpha\beta = k_{21} k_{10} \quad (6)$$

$$\frac{A\beta + B\alpha}{A + B} = k_{21} \quad (7)$$

In the present case these equations may be solved to give

$$k_{12} = 0.2092$$

$$k_{21} = 0.1343$$

$$k_{10} = 0.09198$$

The entire time course of a substance in the plasma, described by a 2 compartmental model, during an infusion and afterwards is described as

$$C = \frac{k_0(k_{12}-\alpha)(1-e^{\alpha T})}{V_c \alpha(\alpha-\beta)} e^{-\alpha t} + \frac{k_0(\beta-k_{12})(1-e^{\beta T})}{V_c \beta(\alpha-\beta)} e^{-\beta t} \quad (8)$$

where k_o is the rate of infusion

V_c is the apparent volume of distribution
of the central compartment

while the infusion continues $T=t$ and varies with time.

When the infusion ceases T becomes a constant corresponding to the time at which the infusion was stopped. When considering the infusion phase only ($T=t$) equation (8) may be simplified

$$C = \frac{k_o}{V_c k_{10}} \left(1 + \frac{\beta - k_{10}}{\alpha - \beta} e^{-\alpha t} + \frac{k_{10} - \alpha}{\alpha - \beta} e^{-\beta t} \right) \quad (9)$$

Given the decay characteristics of l-dopa in sheep plasma it may be shown with equation (9) that a 90 minute infusion will lead to a plasma concentration of 94.6% of the steady state concentrations (C_{ss}). The apparent volume of the central compartment also derived from (9) is

$$V_c = \frac{k_o}{C_{ss} k_{10}} \times 0.946 \times \frac{1}{1000}$$

$$= 1.627 \text{ l}$$

Finally the apparent total volume of distribution of l-dopa in the sheep (volume of the central compartment plus the volume of the peripheral compartment) is defined by

$$V_B = \frac{k_o}{C_{ss} \beta} \quad (10)$$

In the present case this volume is 49.053 l, greatly in excess of the total extracellular fluid volume of the sheep (30% of total body weight) (Hix *et al.* 1953). This indicates concentration of l-dopa perhaps by tissue binding is occurring.

The predicted plasma response curve for l-dopa using equation (8) is plotted in Figure 5-11 alongside the experimental points. The predicted decline in the l-dopa level lags slightly behind

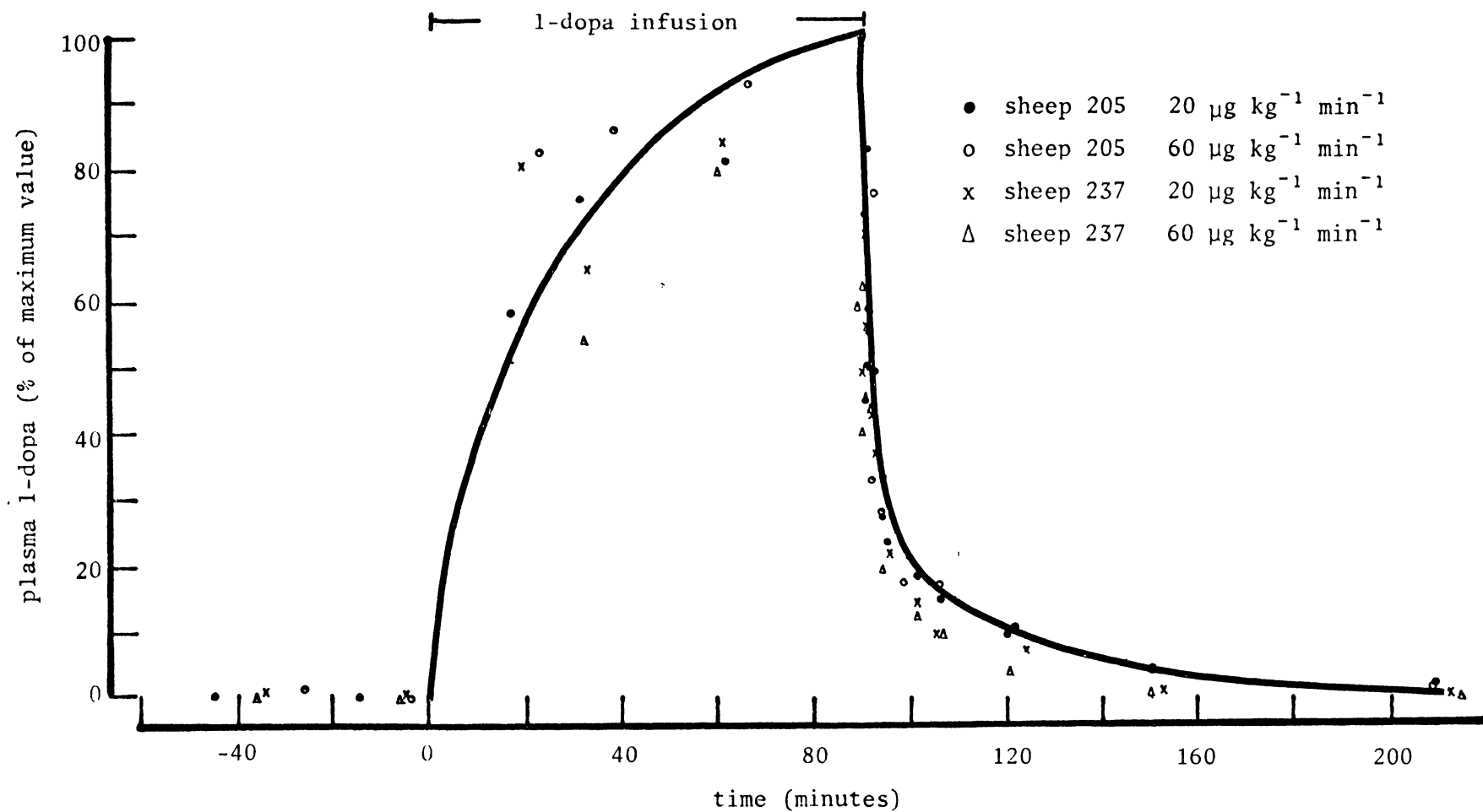


Figure 5-11. Experimental data points from 90 minute l-dopa infusions (scaled to % of maximum value) with the predicted curve from the pharmacokinetic model.

the experimental points. This is a consequence of the interruption in the observed experimental decline in the l-dopa concentrations. Apart from this aspect the fit appears satisfactory.

Free dopamine in modest concentrations ($8-26 \text{ ng ml}^{-1}$) appears in the plasma of Parkinson's disease patients treated with orally administered l-dopa (Tyce *et al.* 1974). Conjugated dopamine levels rise to very high concentrations in the same patients. The high levels of l-dopa in the plasma of the infused sheep made it impossible to quantify the free dopamine in view of the marked interference in the fluorimetric assay by l-dopa (§4.4). Levels of noradrenaline in these sheep were high (up to 9 ng ml^{-1}) during and immediately after the infusion but the noradrenaline assay results should also be viewed with caution. Even though the cross interference of l-dopa in the noradrenaline assay is small (0.12%) a significant and uncertain contribution by the very high l-dopa levels to the noradrenaline assay fluorescence still occurs. Noradrenaline determined radioenzymatically remains low ($<0.5 \text{ ng ml}^{-1}$) (Shoulson *et al.* 1975) or else increases marginally (Ziegler *et al.* 1977) during l-dopa infusions in human subjects. Chromatographic separation is clearly a prerequisite to the study of the other catecholamines in l-dopa infusions where the assay is based on fluorimetry.

While data collected from a single compartment do not permit physical attributes to be assigned to the model components it is reasonable to assume that the very fast initial fall in l-dopa concentration is due to a redistribution phase while the second slower decline represents a mixture of metabolism, excretion and/or slow uptake by nervous and other tissues. There appears to be a small residual level of l-dopa in the plasma 2 hours after exogenous infusion has ceased slightly higher than predicted by the model on the basis of the fitted equations (Figure 5-9a). This may indicate a slow release of l-dopa from binding sites in body tissues.

5.4 OVINE PLASMA LACTIC ACID LEVELS IN CANNULATION, RECOVERY AND DURING EXOGENOUS CATECHOLAMINE INFUSIONS

INTRODUCTION

Lactic Acid in the Blood

Since lactic acid is discussed in conjunction with the catecholamines and cortisol throughout this thesis a brief discussion of the place of lactic acid in carbohydrate metabolism is necessary. Some physiological situations encountered in the work described in this and subsequent chapters are also briefly covered with respect to the participation of blood lactic acid.

Carbohydrates are digested down to glucose which is then phosphorylated to glucose-6-phosphate and converted either to glycogen or to pyruvate via the Embden-Meyerhoff pathway (Figure 5-12). Pyruvate occupies a central position in intermediary metabolism, taking part in fat and protein as well as in carbohydrate metabolism.

Under aerobic conditions most of the pyruvate formed in the glycolytic process is metabolized to carbon dioxide and water through the citric acid cycle. In the absence of oxygen, pyruvate acts as a hydrogen ion acceptor for reduced nicotinamide-adenine dinucleotide (NADH) and is reduced to lactic acid, catalyzed by the lactic dehydrogenase (LDH) system. Thus hypoxia is associated with high circulating levels of lactic acid (Huckabee 1958b). However there are many factors including the catecholamines themselves that may raise blood lactic acid levels independently of a lack of tissue oxygen (Chamberlain & Lis 1968a). In addition blood lactic acid may not be in rapid equilibrium with tissue lactic acid, the canine heart being a case in point (Glaviano 1965).

High levels of lactic acid, over 100 mg dl^{-1} are found in the blood of exhausted animals after maximal exercise (Kluger *et al.* 1972) and are indicative of anaerobic metabolism or at least an inability of muscle cells to make use of the oxygen made

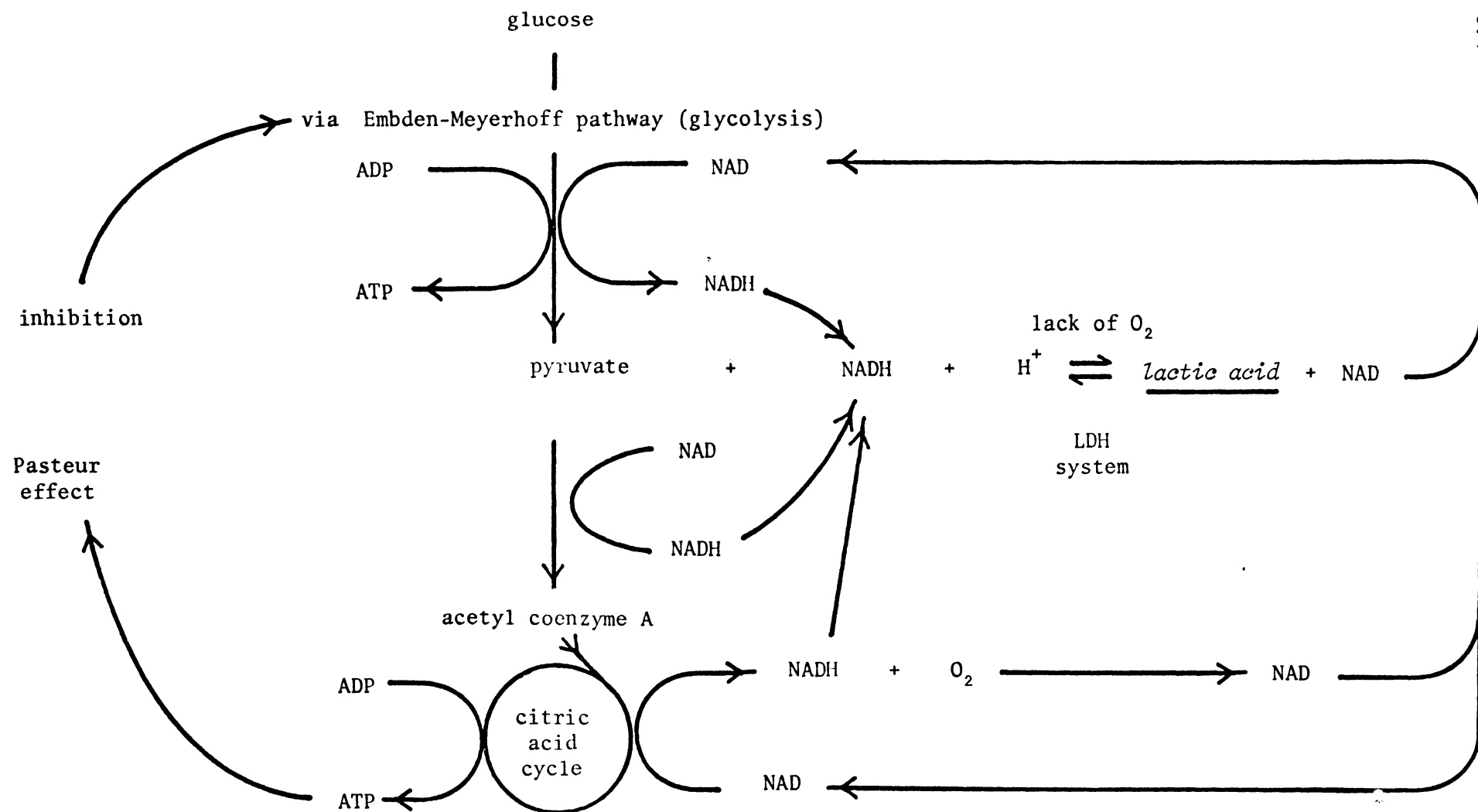


Figure 5-12. Lactic acid in carbohydrate biochemistry.

[from Chamberlain & Lis 1968a]

available (Hubbard 1973). Lactic acid increments do however occur in submaximal work (Hubbard 1973) and levels are reduced in trained compared to untrained animals at any given work intensity (Klausen *et al.* 1974). Marked increases in lactic acid production may not in fact be accompanied by a commensurate increase in plasma levels if utilization also increases at a comparable rate. For example this situation arises in dogs during cold exposure (Minaire *et al.* 1971) but also to some extent during exercise (Hubbard 1973). Adrenaline may also have the effect during exercise of increasing the rate of muscle glucose utilization which tends to negate the concurrent hyperglycaemia (Dill *et al.* 1935). In the sheep the utilization rate of lactic acid (mainly by oxidation to carbon dioxide and water or by conversion to glucose) is roughly linearly related to its blood concentration (Annison *et al.* 1963). Utilization rate declines significantly in sheep starved for 24 hours (Annison *et al.* 1963). These investigators estimated that at least 40% of the lactic acid pool was derived from glucose during the infusion of ^{14}C -glucose. Considerable interconversion between glucose and lactic acid was evident in the sheep but little incorporation of either substance into liver or muscle glycogen was observed. Small diurnal changes ($<10 \text{ mg dl}^{-1}$) have been found in horses with a maximal level occurring around midday (Gill *et al.* 1972).

Blood Lactic Acid in Anaesthesia, Surgery and During Recovery

The presence of elevated lactic acid in the blood of anaesthetized mammals undergoing surgery has been reported under some circumstances. Ether or cyclopropane anaesthesia, also associated with a degree of sympathetic stimulation (§5.2), induces a rise in lactic acid (Greene 1961, Hillidge & Lees 1974). Halothane plus oxygen anaesthesia are not associated with increases in lactic acid or pyruvate where respiration is spontaneous or assisted (Greene 1961, Lowenstein *et al.* 1964, Hillidge & Lees 1974). The lack of a stimulatory influence by halothane on catecholamine release as opposed to ether may account for the difference (§5.2). Even in this case however lactic acid rises

in conjunction with hyperventilation or during recovery from the anaesthetic (Chamberlain & Lis 1968b). Hillidge & Lees (1974) suggested that it is also possible that the accumulation of lactate in ether anaesthesia was caused in part by concurrent hyperglycaemia since increased blood lactic acid as well as pyruvate can be induced by the intravenous infusion of glucose (Huckabee 1958a). Oyama & Takazawa (1971) have suggested that the increased blood levels of growth hormone, lowered insulin and raised cortisol play a part in the increase of glucose during operations under ether anaesthesia.

Effects of the Catecholamines on Blood Lactic Acid

Besides their potent physiological effects the most striking consequences of catecholamine release into the circulation are the mobilization of glycogen via activation of the phosphorylase system of the liver and muscle (Williamson 1975) and the mobilization of lipid stores in adipose tissue by activation of an intracellular triglyceride lipase (Birnbaum & Goodman 1977). These metabolic effects are accompanied by an increase in oxygen consumption. While the effects of catecholamines on carbohydrate and fat metabolism are complex with numerous points of interactions, direct and indirect (Ellis 1956, Himms-Hagen 1967, Williamson 1975) and in many respects species dependent (Ellis 1978) the overall effect of sympathetic stimulation is to increase markedly both glucose and lactic acid in the plasma. Injected adrenaline stimulates muscle and hepatic glycogenolysis for example causing a sustained rise in blood glucose levels (Kral *et al.* 1969, Dechezleprêtre & Lechat 1974, Weiser & Grande 1974). These changes are reinforced by the effects of circulating and sympathetic nervous system catecholamines on insulin and glycogen release. These endocrine mechanisms are not fully understood as they are complicated by the existence of both inhibitory α receptors and excitatory β receptors (Porte 1967, Gerich *et al.* 1974) but the overall effect of sympathetic activation is to increase glycogen release (Harvey *et al.* 1974) and decrease insulin release (Porte 1967, Miller 1975). The breakdown of glycogen or glucose to lactic acid consists of a long series

of reactions. Adrenaline in particular stimulates this conversion apparently via its effects on the formation, breakdown and functions of cyclic 3', 5'-adenosine monophosphate within the various target tissues. Muscle glycolysis has been thought to be the major source of blood lactic acid following adrenaline administration (Hynie *et al.* 1961) but recent data from the rat suggests that blood glucose is the principal lactate precursor (Kusaka & Ui 1977). Other adrenergic mechanisms that may reinforce this biochemical modulation include tissue hypoxia due to vasoconstriction and a reduction in blood flow to the liver and other tissues involved in lactic acid uptake (Laborit *et al.* 1967, Eldridge 1974).

While glycaemic responses to catecholaminergic antagonists and agonists are complex it is currently assumed that the effect of injected adrenaline on lactic acid is a β -adrenergic function and is related to simultaneous increases in plasma free fatty acid and glucose concentrations and increased glycogenolysis in muscle, or both, since inhibition of any of these responses also reduces blood lactic acid concentration (Himms-Hagen 1967, Kral *et al.* 1969). The acidosis caused by exogenous catecholamines in the rat is also correlated to β -adrenergic activity and may be prevented with the β blocking drug propranolol (Atkinson *et al.* 1972). The responses to noradrenaline may however involve α receptors as its effects in the rat are potentiated by propranolol and eliminated by the α antagonist phenoxybenzamine (Atkinson *et al.* 1972). The physiological basis for the potentiating effect of propranolol has not been elucidated, but it may be related to the ability of propranolol to inhibit the noradrenaline reuptake mechanism (von Euler & Lishajko 1968). Physiological stimulation such as haemorrhagic shock (Halmagyi *et al.* 1968) or exercise (Juchmès *et al.* 1973) causes a rise in lactic acid that is resistant to propranolol. Even combined α and β blocking is only partially effective in abolishing haemorrhage lactacidaemia (Halmagyi *et al.* 1968).

While both physiological stressors and administered adrenaline may precipitate some common metabolic events including lactic acid production the respective response profiles may differ in both magnitude and timing. Dechezleprêtre & Lechat (1974) found for example that rats subjected to a 5 minute cold stress exhibited a faster and shorter-lived elevation in lactic acid than the rises which followed subcutaneous adrenaline in doses between $0.015\text{--}0.5\text{ mg kg}^{-1}$. An interesting aspect of the metabolic inter-relationships between the catecholamines and lactic acid is the suggestion that lactic acid itself may inhibit adrenal catecholamine release (Juchmès & Cession-Fossion 1971) although there is also some evidence to support a stimulatory effect on adrenal adrenaline output by acidosis (Darby & Watts 1964).

Both noradrenaline and adrenaline have potent effects in increasing free fatty acid and glucose levels in sheep (Luthman & Holtenius 1972) and goats (Radloff & Schultz 1966). Less information however is available on lactic acid-catecholamine interactions in such domestic animals. In lambs exposed to severe cold, lactic acid increased in association with plasma glucose, with smaller increases in free fatty acids (Alexander *et al.* 1968). All three substances were raised by noradrenaline and adrenaline infusions. Adrenaline infused at rates between $1.0\text{ }\mu\text{g kg}^{-1}\text{ min}^{-1}$ and $10.0\text{ }\mu\text{g kg}^{-1}\text{ min}^{-1}$ for up to 30 minutes produced a mean increase in lactic acid concentration of 41 mg dl^{-1} while noradrenaline at $3.3\text{--}10.0\text{ }\mu\text{g kg}^{-1}\text{ min}^{-1}$ produced mean lactic increments of 19 mg dl^{-1} . Phentolamine hydrochloride (α receptor blocking agent) had no effect on lactic acid in cold exposure but did reduce the glycaemic and free fatty acid responses. Propranolol (β receptor blocking agent) reduced the magnitude of all metabolic changes observed in cold exposure.

Bassett (1970) reported that 1.5 mg of adrenaline injected intravenously in sheep caused a rise in lactic acid concentrations from 5.3 ± 3.2 ($n=6$) mg dl^{-1} to $33.7\pm 4.2\text{ mg dl}^{-1}$ which was followed by a slow decline to $9.5\pm 4.2\text{ mg dl}^{-1}$ over the subsequent 105 minutes. The same amount of adrenaline infused over 30 minutes

(50 $\mu\text{g min}^{-1}$) caused a greater and more prolonged lactic acid peak (maximum level $55.9 \pm 8.3 \text{ mg dl}^{-1}$) at 15 minute postinfusion. Even 90 minutes postinfusion the plasma lactic acid was still $32.0 \pm 8.6 \text{ mg dl}^{-1}$. In an adrenaline dose response infusion study the increment in lactic acid was shown to be linear with the log dose over the range 2.29-45.8 $\mu\text{g min}^{-1}$. Noradrenaline infusion at the rate of 4.23 $\mu\text{g min}^{-1}$ for 60 minutes had little effect on lactic acid concentration although free fatty acid levels were increased. The α receptor blockers phentolamine and phenoxybenzamine did not alter the lactic acid responses to adrenaline or isoprenaline infusions but propranolol abolished their effects on lactic acid and free fatty acids (Bassett 1970). In the pig plasma lactic acid increased from a preinfusion value of $6.3 \pm 3.1 \text{ mg dl}^{-1}$ ($n=5$) to $105 \pm 31 \text{ mg dl}^{-1}$ at 10 minutes following a 50 minute adrenaline infusion ($1 \mu\text{g kg}^{-1} \text{ min}^{-1}$) and then decreased toward preinfusion values (Baetz *et al.* 1973). Noradrenaline ($1 \mu\text{g kg}^{-1} \text{ min}^{-1}$) was without effect.

EXPERIMENTAL METHODS - See §5.2

EXPERIMENTAL RESULTS AND DISCUSSION

Cannulation and Recovery

The lactic acid levels observed in the cannula implantation of the present study (up to 31.6 mg dl^{-1}) (Figure 5-13) are high especially when the fact that halothane anaesthesia was employed is taken into account. Nevertheless the conditions of the present surgery differ in important respects from those described by Lowenstein *et al.* (1964) in the human and Hillidge & Lees (1974) in the horse. In contrast to these studies sympathetic activation prior to induction of anaesthesia was accentuated. First the sheep were maintained indoors, separated from their flock, in a strange environment for 24 hours without food before a restraint stress (accompanied by muscular exertion) for the intravenous injection of the premedication. Restraint combined with venipuncture produced elevated lactic acid levels in a subsequent experiment (§6.7). Thus the lactic acid level in the blood may simply reflect the effect of these stressors earlier

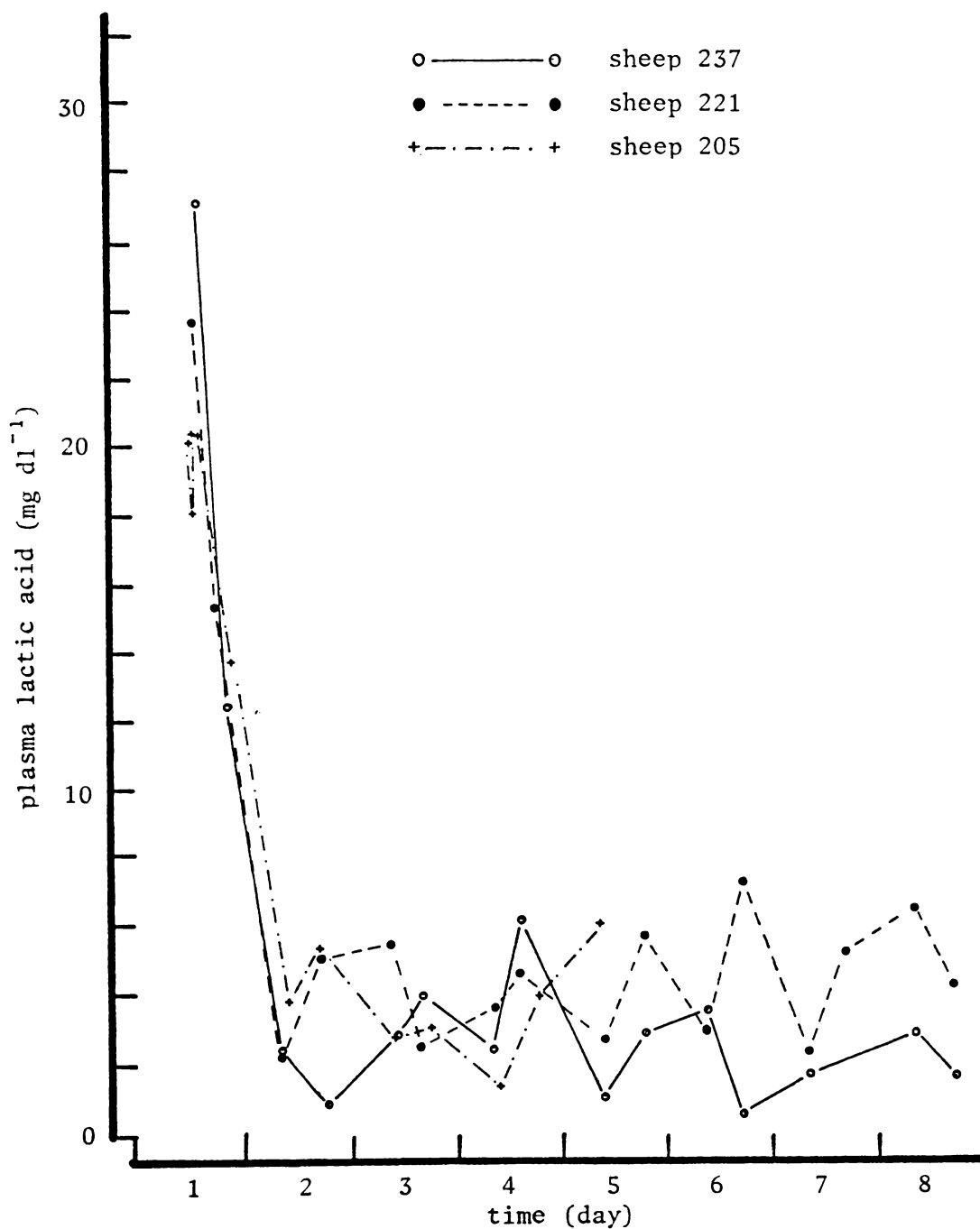


Figure 5-13a. Plasma lactic acid during and shortly after cannulation (day 1) and during recovery (days 2-8). Over the recovery period samples were collected twice daily from patent cannulae.

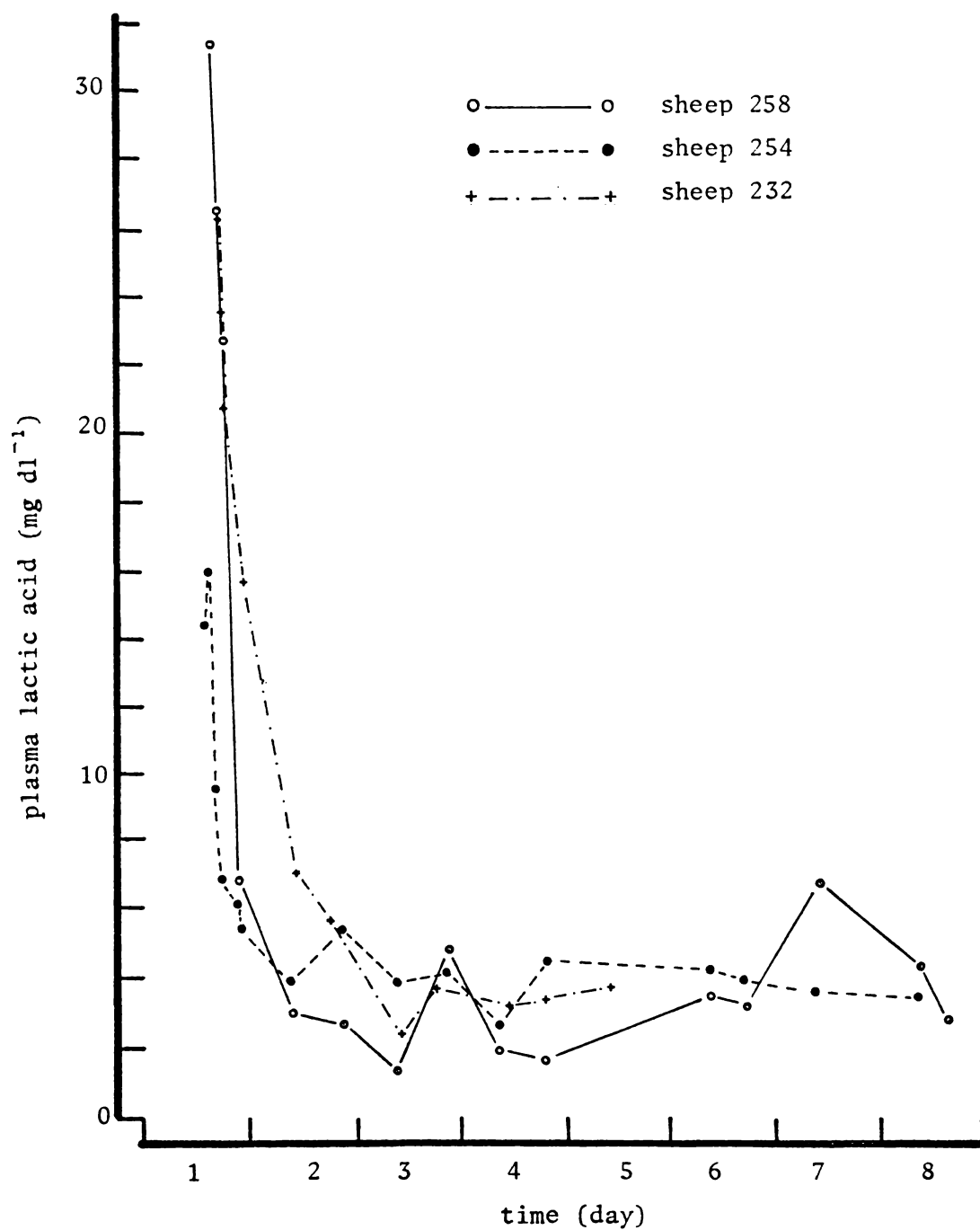


Figure 5-13b. Plasma lactic acid during and shortly after cannulation (day 1) and during recovery (days 2-8). Over the recovery period samples were collected twice daily from patent cannulae.

in the day and especially in the preceding 30 minutes. In addition catecholamine levels were elevated during the operation (§5.2) and it should be noted that the anaesthesia was maintained as light as possible for superficial surgery. Tissue hypoxia in this situation seems unlikely.

After the operation lactic acid levels fell steadily and were essentially at baseline over the following day ($4.3 \pm 2.2 \text{ mg dl}^{-1}$, $n=12$) and no significant changes were seen subsequently before commencement of the catecholamine infusions. The differences in the lactic acid responses between individual sheep ($F_{5,46} = 5.81$) and between lactic acid levels on day 1 and days 2-8 ($F_{1,46} = 157.9$) were both highly statistically significant ($p < 0.001$).

Catecholamine Infusions

The overall baseline level of lactic acid ($4.4 \pm 1.7 \text{ mg dl}^{-1}$, $n=31$) in the 4 saline infusion series of the present study (Figure 5-14a) is similar to that seen in the same sheep in the days following cannulation and to resting levels reported by Bassett (1970) in sheep ($5.3 \pm 3.2 \text{ mg dl}^{-1}$, $n=6$). Neither the introduction of l-dopa nor dopamine had any effect on plasma lactic acid (Figure 5-14b,c) ($P > 0.2$, 2-way analysis of variance). Although dopamine has both α and β stimulating effects on the cardiovascular system (§5.2) (albeit partly an indirect effect exerted via noradrenaline) it lacks any metabolic effects on lactic acid in the doses used in the present study. The infusion of l-dopa at $60 \text{ } \mu\text{g kg}^{-1} \text{ min}^{-1}$ appears to increase slightly the variability of the lactic acid ($F_{3,8} = 2.87$, $p < 0.2$, 2-way analysis of variance). If this effect is a real one, and more data need to be collected at higher dose rates, then the cause may be higher circulating noradrenaline due to the metabolic conversion of l-dopa (§5.3).

Noradrenaline infused at 0.33 and $1.00 \text{ } \mu\text{g kg}^{-1} \text{ min}^{-1}$ has a small effect on plasma lactic acid (Figure 14d) ($F_{3,7} = 3.32$, $P < 0.10$, 2-way analysis of variance). Adrenaline, by a considerable margin, has a much greater effect than noradrenaline (Figure 14e).

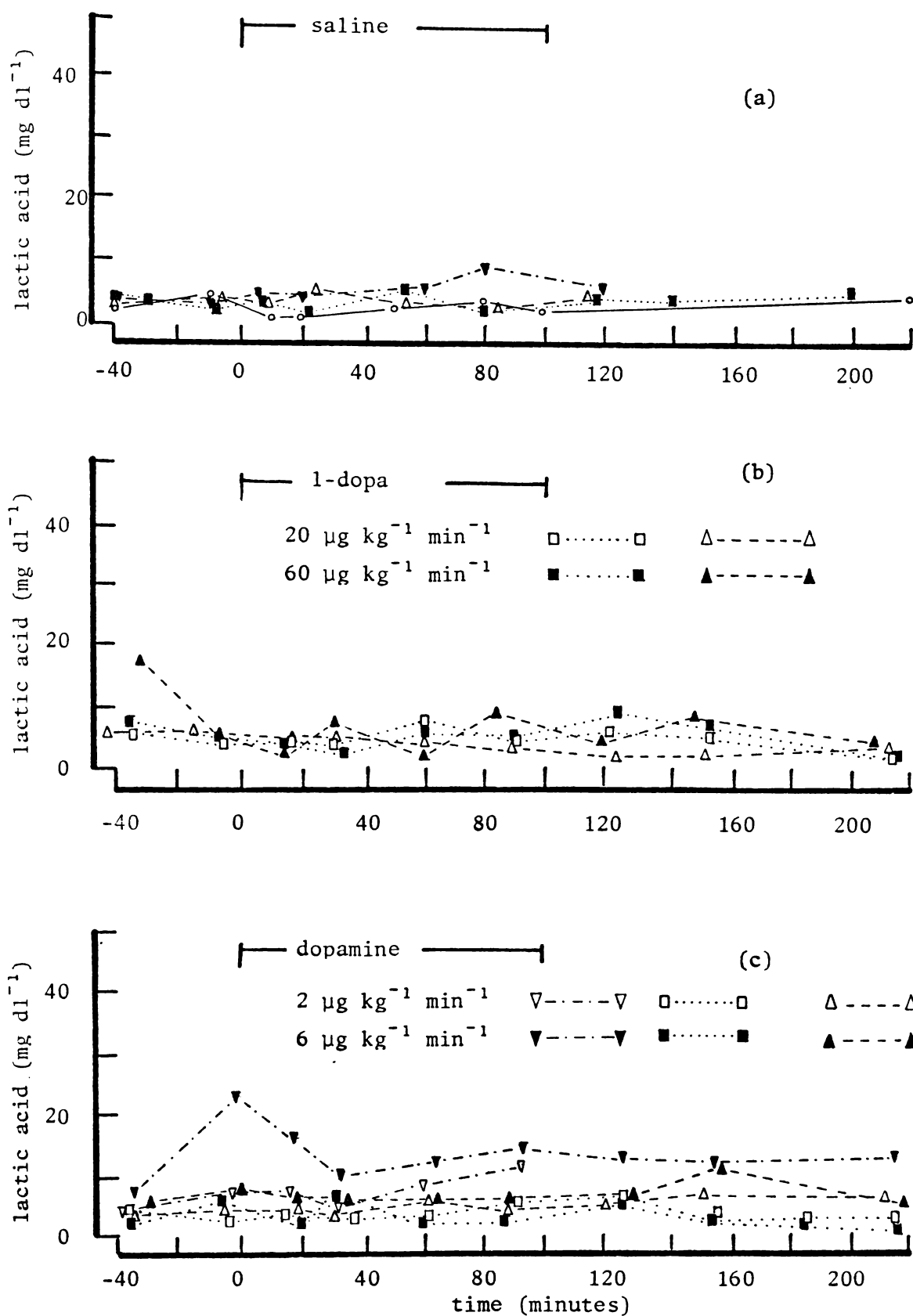
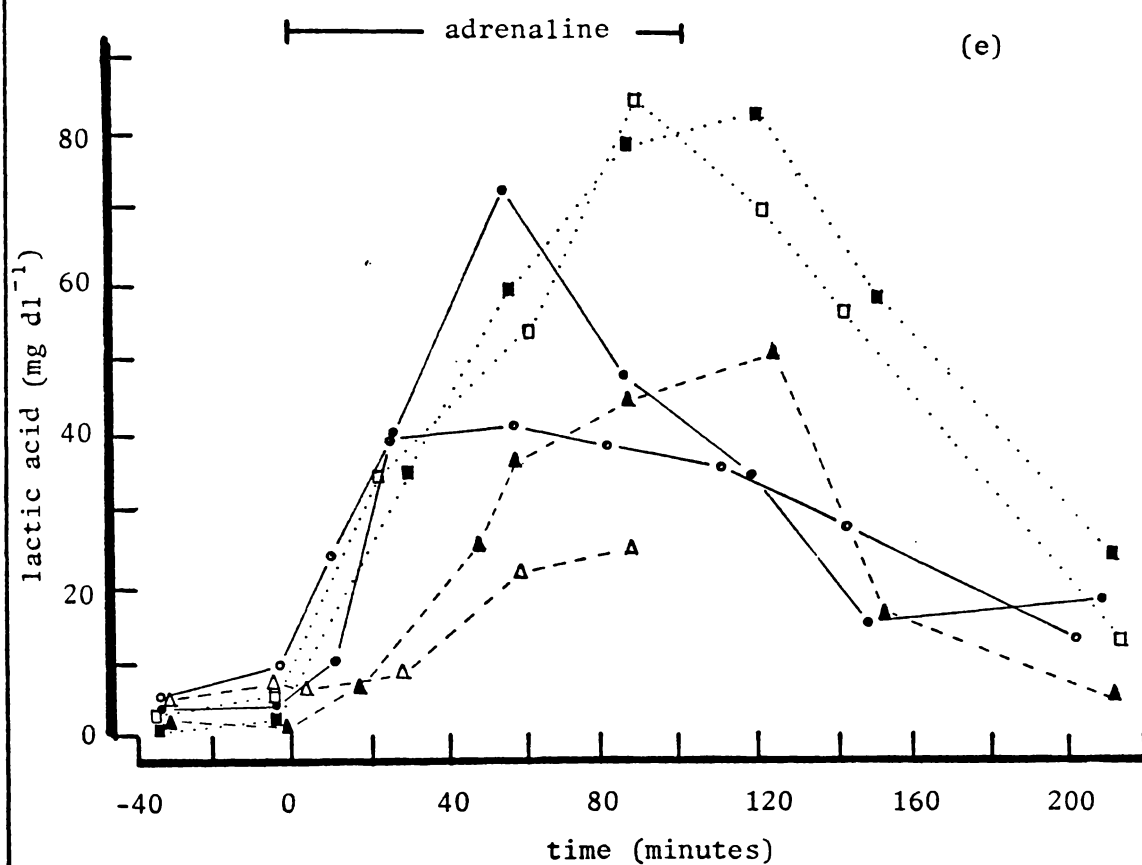
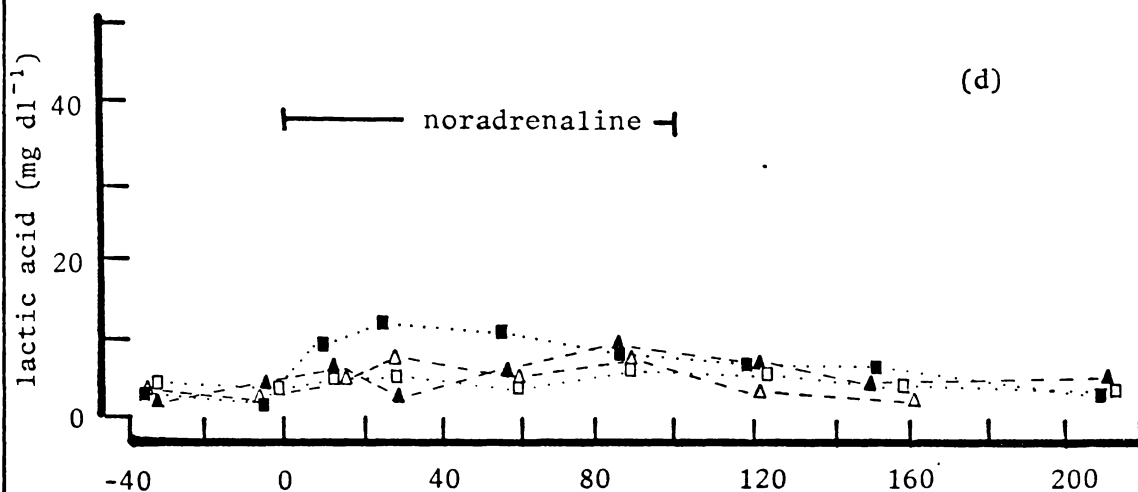


Figure 5-14 (a)-(c). Plasma lactic acid in 4 sheep during 90 minute infusions of saline, l-dopa and dopamine.

sheep 205 Δ $\blacktriangle$ sheep 221 ∇ $\blacktriangledown$
 sheep 237 $\square$ $\blacksquare$ sheep 254 $\circ$ $\bullet$



sheep 205	Δ ----- Δ ($0.33 \mu\text{g kg}^{-1} \text{min}^{-1}$)	$\blacktriangle$ ----- $\blacktriangle$ ($1.00 \mu\text{g kg}^{-1} \text{min}^{-1}$)
sheep 237	$\square$ $\square$ ($0.33 \mu\text{g kg}^{-1} \text{min}^{-1}$)	$\blacksquare$ $\blacksquare$ ($1.00 \mu\text{g kg}^{-1} \text{min}^{-1}$)
sheep 254	$\circ$ ——— $\circ$ ($0.33 \mu\text{g kg}^{-1} \text{min}^{-1}$)	$\bullet$ ——— $\bullet$ ($1.00 \mu\text{g kg}^{-1} \text{min}^{-1}$)

Figure 5-14 (d)-(e). Plasma lactic acid in 3 sheep during 90 minute infusions of noradrenaline and adrenaline.

A maximum level of 83.8 mg dl^{-1} was achieved. Some difference is discernible in the lactic acid responses to the 2 rates of infusion in 2 of the animals; the third showed no difference. High lactic acid levels were maintained after the infusion cessation in 2 sheep and indicate a continued production of lactic acid, impaired removal or both, since in man for example, a turnover time of 18.4 minutes ($n=4$) has been reported (Searle & Cavalieri 1972). In the presence of adequate circulation and under conditions of rest blood lactic acid is removed by uptake processes at a rate closely but not quite linearly related to the blood concentration (Eldridge *et al.* 1974). The high post-infusion levels of adrenaline in the blood (55.2) may be partly responsible for sustained lactic acid levels in some sheep. Other infusion studies in sheep have produced lactic acid concentrations similar to those in the present study both when the infusion rate was similar (Bassett 1970) or higher (Alexander *et al.* 1968).

5.5 OVINE PLASMA CORTISOL LEVELS IN CANNULATION, RECOVERY AND DURING EXOGENOUS CATECHOLAMINE INFUSIONS

INTRODUCTION

Cortisol in the Blood

The steroid hormones of the adrenal cortex are broadly classed into those having effects on sodium and potassium metabolism (mineralocorticoids) and those whose predominant effects are on carbohydrate and protein metabolism (glucocorticoids). Among the glucocorticoids, cortisol and corticosterone are the dominant members in the sheep and of these cortisol is pre-eminent (Bush & Ferguson 1953). Glucocorticoid synthesis and release is largely under pituitary corticotrophin (adrenocorticotrophic hormone or ACTH) control. In turn corticotrophin is regulated by a small hypothalamic peptide (corticotrophin releasing hormone or CRH) conveyed to the anterior pituitary via the hypophyseal portal vessels (Reichlin 1974). Above this are fairly well defined

groups of neurones situated mainly in the median eminence which are especially sensitive to neurogenic impulses relayed from the cerebral cortex and other areas of the brain (Yates *et al.* 1971, Gann *et al.* 1977b). The action of corticotrophin depends upon its ability to stimulate the adrenal cortices to increase their output of corticosteroids (Johnston 1972) which, in turn, are responsible for widespread metabolic effects (including gluconeogenesis) (Leung & Munck 1975). A great variety of environmental and internal stimuli are capable of increasing levels of plasma corticosteroids (Kilgour & de Langen 1970, Gann & Cryer 1973, Falconer 1976) but habituation to a specific stressor such as the effect of the presence of dogs on sheep (Falconer 1976) or repeated exposure of cats to an unfamiliar environment (Redgate *et al.* 1974) leads to an extinction of the corticosteroid response. The resulting elevated blood and tissue concentration of corticosteroids appears to be essential for the animal to withstand the stressor. The overall mechanism for the control of corticosteroid release is highly integrated and complex with many possible inputs to the system both neural and humoral (Gann & Cryer 1973, Sayers & Portanova 1975).

It has been recognized over the last decade that corticosteroid secretion is more intermittent than continuous in character. Even under conditions of minimal stimulation the release of corticosteroids is pulsatile and concentrations may vary markedly over short periods of time (Hellman *et al.* 1970). The cortisol profile of a human male monitored over 24 hours at 20 minute intervals revealed 8 periods of active secretion, occupying about 25% of the total time, separated by long quiescent periods (Hellman *et al.* 1970).

In the sheep cortisol kinetics may be described in terms of a 2 compartmental model similar to that developed for l-dopa in §5.4 (Paterson & Harrison 1968, Panaretto & Vickery 1972). Patterson & Harrison (1968) estimated half-lives of 1-4 minutes and 19 minutes for the fast and slow exponential decay curves respectively. In humans the disappearance of cortisol is rather slower

(half-lives of 6 and 68 minutes in the study of Hellman *et al.* (1970)).

Circadian Rhythm The secretion of cortisol is subject to a circadian variation in many species (Atcheson & Tyler 1975). Bassett (1974) found no significant fluctuations in cortisol levels with the time of the day nor with the feeding of sheep housed indoors, but these results are contradicted by McNatty *et al.* (1972) who found a circadian rhythm with a maximum secretion at 9.30 am in housed sheep fed *ad libitum*. Holley *et al.* (1975) confirm the existence of a rhythm with maximal secretion at 4 pm and 4 am. The afternoon peak however was coincident with the once-daily feeding session.

Corticosteroid Secretion Latencies A perceivable time lag occurs between a stimulus known to activate the pituitary-adrenal axis and the first measurable increase in the plasma corticosteroid level. In the rat secretion of corticotrophin releasing hormone follows stimulation very quickly (Hiroshige *et al.* 1971) and corticotrophin release occurs within 2½ minutes (Jones *et al.* 1972). In turn the adrenal response to corticotrophin is rapid and expressed as an increase in adrenal cyclic adenosine monophosphate content which reaches a maximum within 3 minutes accompanied by an increase in steroid secretion (Liddle 1974). These stages result in an overall stimulus-secretion delay of the order of 3 minutes for the appearance of augmented corticosterone in rat plasma (Jones *et al.* 1972). Delays of the same magnitude have been reported between corticotrophin injection and corticosteroid responses in man (2.9-6.7 minutes) (McDonald *et al.* 1969b) and sheep (2-3 minutes) (Beaven *et al.* 1964). Maximum corticosteroid levels in sheep occur 5-11 minutes after corticotrophin injection (Beaven *et al.* 1964) and 30-40 minutes after various stressors (Kilgour & de Langen 1970). The dose level of corticotrophin (McDonald *et al.* 1969b) and the nature and duration of the stressor (Kendall *et al.* 1972, Feldmann & Brown 1976, Gann *et al.* 1978) will however modify these responses.

The delayed and dynamically labile nature of corticosteroid secretion modulated by complex control mechanisms is in contrast to the almost immediate nervously-activated and pulsatile catecholamine release. Cognizance of this difference is essential to the design of experiments and to the interpretation of data when both these hormone groups are monitored.

Feedback Inhibition of Corticotrophin Release by Corticosteroids

Feedback control of corticotrophin secretion by corticosteroids has been demonstrated both by the chronic increase in corticotrophin secretion which follows adrenalectomy (Dallman *et al.* 1972) and by the inhibition of corticotrophin secretion after corticosteroid treatment (Jones *et al.* 1972). The physiological significance of this effect remains obscure however since many stressor-induced rises in corticosteroid plasma levels do not appear to affect the responses to later additional stimuli (Dallman & Jones 1973, Dallman & Wilkinson 1977). Dallman and associates concluded that an initial stimulus to the hypothalamic-pituitary axis may act to cause a period of hyper-responsiveness of the corticotrophin-releasing system that offsets the inhibition caused by high circulating corticosteroid levels. The complexities of the overall system arise because of the different magnitudes and timing of the stimulation, facilitation and feedback effects in any given situation. The actual observed response depends on the outcome of these interactions.

Biphasic Response of Corticosteroids to Stimuli

Corticosteroid secretion in response to a severe stress may exhibit a biphasic pattern with a high initial release rate followed by a fall and then a period of sustained release. This is seen in sheep following cannulation (Espiner *et al.* 1972) when high secretion rates 10-20 minutes after jugular cannulation fall to close to zero at 40-60 minutes with a rebound to high output levels lasting for about 4 hours. A similar pattern in corticosteroid concentration is seen in rats after scalding or immobilization (Knigge *et al.* 1959) excepting that the biphasic responses occur over a much longer time span, especially in the case of scalding.

Investigations of corticotrophin releasing hormone after ether-laparotomy stress (Hiroshige *et al.* 1971) and of corticotrophin after unilateral or bilateral adrenalectomy (Brodish & Long 1956) have shown dynamic changes consistent with those observed in the corticosteroids. A single injection of corticotrophin causes a significant elevation of plasma 17-hydroxycorticosteroids in calves at 40 minutes, immediately followed by a drop in levels towards baseline at 60-70 minutes and a return to highly significantly elevated levels between 100 minutes and 8 hours. A second corticotrophin injection at 30 minutes prevented the drop seen after the single injection at 60-70 minutes (Shaw & Nichols 1963).

The basis of the phenomenon has not been agreed upon since the control of corticotrophin release itself is still controversial (Hodges 1976). Depletion of pituitary corticotrophin stores has been ruled out in view of the comparatively small amount released in relation to total pituitary reserves (Espiner *et al.* 1972) and the ability of the pituitary to respond to a subsequent stressor (Hiroshige *et al.* 1971). A biphasic release of corticotrophin is consistent with the existence of dual and temporally distinct feedback inhibition mechanisms as proposed by Yates *et al.* (1971). Under this scheme the initial spurt in cortisol secretion is thought to activate a rapid acting rate-inhibitory component (that is a rapidly *increasing* level of blood corticosteroids inhibits further secretion) of brief duration whereas the subsequent fall after the second peak could represent a more slowly acting and delayed proportional (to corticosteroid concentration) component of the feedback mechanism. The interplay of these 2 feedback operations separated by a long delay could thus result in the multiphase response to stimuli. Saturation of the fast feedback mechanism occurs when corticosteroids in the blood reach high (but endogenous) concentrations and no inhibition of further stressor corticosteroid responses is observed (Jones *et al.* 1972). Some anatomical specificity for a dissociation between the fast and slow release of corticotrophin has been demonstrated in the cat brain (Redgate 1970). The data of Hiroshige *et al.* (1971) further suggest that the

initial stimulus evokes a fast corticotrophin releasing hormone-dependent phase of corticotrophin release followed by a second phase in which corticotrophin releasing hormone plays a lesser role. At the same time the hypothalamus retains the ability to react to a new stimulus by secretion of further corticotrophin releasing hormone.

Cortisol in Anaesthesia, Surgery and Recovery

Most reported measurements of cortisol related to surgical stress have been in humans. These results have shown that both corticotrophin (Newsome & Rose 1971) and cortisol (Charters *et al.* 1969, Hamanaka *et al.* 1970) are significantly augmented during surgical operations. For example in a group of 6 patients during surgery under general anaesthesia plasma corticotrophin rose from $31 \pm 25 \text{ pg ml}^{-1}$ to $256 \pm 169 \text{ pg ml}^{-1}$ between the preoperative control sample and another 1 hour following the skin incision during the operative procedure (Newsome & Rose 1971). The anaesthetic alone did not alter the corticotrophin concentration. Matsuki & Oyama (1971) confirm that halothane anaesthesia does not alter cortisol in the plasma of human patients. Newsome & Rose (1971) also noted a complete abolition of both growth hormone and corticotrophin responses to surgery when the operation involved spinal anaesthesia, indicating an afferent pathway via the spinal cord for corticotrophin release. Other anterior pituitary hormones (growth hormone, follicle stimulating hormone, luteinizing hormone and thyroid stimulating hormone) as well as cortisol were examined by Charters *et al.* (1969) before, during and after surgery in humans. Cortisol concentrations in 6 of the 7 patients increased during surgery from a control concentration of $127 \pm 22 \text{ ng ml}^{-1}$ to $243 \pm 120 \text{ ng ml}^{-1}$ 1 hour after starting the operative procedure and remained high throughout the surgery ($292 \pm 125 \text{ ng ml}^{-1}$ at 2 hours). Cortisol was still elevated in the serum of the patients on the first postoperative day but returned to the normal range for the remainder of the convalescence. Growth hormone but not thyroid stimulating hormone was significantly increased as well. Follicle-stimulating and luteinizing hormone concentrations were depressed during the

postoperative convalescence. Hamanaka *et al.* (1970) using a fluorimetric cortisol assay found significant elevations in cortisol levels, also accompanied by a high corticosterone concentration following anaesthesia, during, and after surgery. The maximum concentration of plasma cortisol occurred 2 hours after the end of the operation. Five to 6 days elapses before cortisol returned to control values. It should be noted that the secretion rate of cortisol during surgery may well be underestimated by reliance on changes in the plasma concentration, since large increases in both the volume of distribution (175%) and the metabolic clearance rate (130%) occur during and after surgery in humans (Kehlet & Binder 1973).

Effects of the Catecholamines on Corticosteroid Release

Considerable evidence has been amassed to suggest that the catecholamines play an important role in the regulation of anterior pituitary hormone secretion, particularly growth hormone and corticotrophin. However the evidence is not in general agreement on the direction or the nature of this modulation nor on the significance, if any, of systemic catecholamines (in the physiological range) for corticosteroid release. The high dopamine and noradrenaline contents of the hypothalamus (Fuxe & Hökfelt 1969) suggest that these catecholamines could be significant in the regulation of anterior pituitary secretion. Two different approaches to the study of catecholamine-pituitary interactions have been utilized. The first involves the correlation of turnover rates of dopamine or noradrenaline in the central nervous system, as determined by histochemical means, with the physiological or pharmacological inputs which alter endocrine function. The second involves the administration by various routes of the catecholamines themselves or of inhibitors of catecholamine synthesis, and measurement of the endocrine responses. These techniques have their limitations and the resulting data are sometimes in conflict.

McDermott *et al.* (1950) showed that pituitary graft tissue in rats responded to adrenaline by releasing corticotrophin (inferred by adrenal ascorbic acid depletion) and Krieger & Krieger (1970) reported that the implantation of noradrenaline into certain areas of the cat brain produced an acute rise in plasma corticosteroid levels. Toivola & Gale (1972) also found a rise in the corticosteroid levels after noradrenaline introduction into the hypothalamus of baboons. On the contrary van Loon and his associates have produced pharmacological evidence for an inhibitory catecholaminergic, and in particular a noradrenergic, action on corticotrophin release in the dog (van Loon *et al.* 1971a, van Loon *et al.* 1971b, Scapagnini *et al.* 1972), a concept supported by the *in vitro* studies of Jones *et al.* (1976) but disputed by others also on pharmacological grounds (Kaplanski *et al.* 1973, Lippa *et al.* 1973). The failure of 6-hydroxydopamine through neural destruction in rats (Kaplanski *et al.* 1974) or of peripherally administered reserpine through neural catecholamine depletion in sheep (Purchas 1973) to influence pituitary-adrenal function is not definitive evidence against catecholamine regulation of the axis since complete pharmacological blockage of highly redundant nervous pathways is difficult to achieve (Ulrich & Yuwiler 1973).

The effects of intravenous catecholamine administration on corticosteroid levels are also inconsistent. Species differences may be important in this regard and the question of circulating catecholamine access to brain structures is also a consideration. Noradrenaline (Weil-Malherbe *et al.* 1961) and adrenaline (Weil-Malherbe *et al.* 1959) entry to the central nervous system from the periphery is restricted by the blood brain barrier but considerable uptake of systemic catecholamines into the hypothalamus and pituitary does occur (Axelrod *et al.* 1959b, Weil-Malherbe *et al.* 1961, Lichtensteiger & Langemann 1966). L-dopa readily crosses the blood brain barrier (Bertler & Rosengren 1959, Lichtensteiger & Langemann 1966) and can thus influence the catecholamine content and activity of central adrenergic neurones.

The α -adrenergic stimulating agent methoxamine causes a rise in plasma corticotrophin and cortisol in humans, an effect that is significantly blunted by the simultaneous administration of phentolamine (Nakai *et al.* 1973). The plasma corticotrophin response to insulin-induced hypoglycaemia tested during phentolamine infusion was partially but significantly inhibited compared with controls treated only with insulin and saline. Beta blockage with propranolol enhanced the plasma corticotrophin response to hypoglycaemia (Nakai *et al.* 1973).

Peripheral catecholamines, at least in pharmacological doses, can increase corticosteroid and corticotrophin levels in the blood. Van Loon *et al.* (1971b) in fact draw a distinction between the central inhibitory and the peripheral excitatory effects of catecholamines, reporting that both l-dopa and noradrenaline caused rises in corticosterone $\frac{1}{2}$ -1 hour following the administration of these substances to rats. L-dopa has no effect on basal or metyrapone-stimulated corticotrophin release in man but it markedly inhibits stress-induced release in dogs and cats (van Loon *et al.* 1971a, Lebovitz *et al.* 1973) as does dopamine in rats in doses that do not cause a significant rise in arterial pressure (King 1969). Corticotrophin has been reported to be increased in normal human subjects by l-dopa administered orally (Simonin *et al.* 1972). On the other hand a fall (Balestreri *et al.* 1974a) and no change (Hayek & Crawford 1972) have been found in blood cortisol concentration in man following oral l-dopa. In another human study (Wilcox *et al.* 1975) dopamine and noradrenaline infused intravenously (in increasing doses until a 30% increase in systolic blood pressure had occurred) resulted respectively in an increase (though statistically non-significant) and a decrease in both plasma corticotrophin and cortisol. Intravenous l-dopa infusions caused significant and consistent rises in both corticotrophin and cortisol. The human cortisol and growth hormone secretion elicited by insulin-induced hypoglycaemia was significantly inhibited by a 150 minute adrenaline infusion ($6 \mu\text{g min}^{-1}$)

initiated 60 minutes before the commencement of the insulin infusion (Müller-Hess *et al.* 1974).

Several recent studies (McNatty & Thurley 1973, Jones *et al.* 1977) have demonstrated a corticosteroid response to exogenous adrenaline administered to sheep. McNatty & Thurley (1973) showed in 3 sheep that intravenous injections of 1, 2 or 3 mg of adrenaline caused peaks of cortisol rising in magnitude with the adrenaline dose to a mean maximum of 150 ng ml^{-1} at 30 minutes. An indication of a biphasic peak response to 3 mg of adrenaline was also observed. The cortisol release in response to adrenaline diminished with the length of time the sheep were housed and sampled. In one of two sheep injected with noradrenaline an increase in plasma cortisol occurred. The order of potency of corticosteroid releasing amines was tyrosine and 1-dopa < dopamine < noradrenaline < adrenaline. Adrenaline infused at the rate of $1.3 \text{ } \mu\text{g min}^{-1}$ into sheep fetuses caused a rise in corticotrophin from $253 \pm 73 \text{ pg ml}^{-1}$ to $1161 \pm 373 \text{ pg ml}^{-1}$ ($n=4$) during a 2 hour infusion (Jones *et al.* 1977) but only one foetus exhibited any change in corticosteroid levels (from 61 ng ml^{-1} to 172 ng ml^{-1}). In 4 pregnant sheep the effect of an adrenaline infusion at the rate of $12.7 \text{ } \mu\text{g min}^{-1}$ for 2 hours (raising the plasma concentration of adrenaline from $<0.05 \text{ ng ml}^{-1}$ to $0.6\text{--}8.5 \text{ ng ml}^{-1}$) was to increase both circulating corticotrophin and corticosteroids. Above 1 ng ml^{-1} of plasma adrenaline there was a linear relationship between the rise in the corticotrophin and the adrenaline plasma concentration. The maternal adrenaline infusion caused a significant ($p < 0.001$) rise in the foetal corticotrophin level from 227 ± 73 to $536 \pm 48 \text{ pg ml}^{-1}$ after 30 minutes which subsequently declined but was still high at 2 hours ($379 \pm 82 \text{ pg ml}^{-1}$). The possibility of a redistribution in cardiac output as a result of the infused adrenaline causing a decrease in the clearance rate of corticotrophin was considered but rejected. Increases in the blood flow to the skeletal muscle and liver that are caused by adrenaline would be expected to increase rather than decrease the clearance rate.

The nature of the adrenergic influence on corticotrophin release is uncertain since the functioning of the hypothalamic-pituitary-adrenal axis may be influenced at many points and other neurotransmitters including acetylcholine and serotonin are involved (Jones *et al.* 1976). There is also evidence (Guillemin 1955) that the effects of peripheral catecholamines are secondary, non-specific effects, a view supported by Naumenko (1967). Peripheral catecholamines are not an essential part of the corticosteroid stimuli in rats since normal response profiles are obtained in the presence of adrenergic or cholinergic blocking agents (Guillemin 1955). Whether secreted catecholamines can significantly alter corticosteroid responses in sheep under natural conditions has still to be demonstrated. Even the lower dose of 1 mg of adrenaline used by McNatty & Thurley (1973) would produce a transient blood adrenaline concentration well in excess of that encountered under most circumstances even in the presence of highly activating stimuli.

Effects of Blood Pressure on Corticosteroid Release

The profound hypotension found in acute adrenal insufficiency and the hypertension found in Cushing's syndrome have long suggested a relationship between circulating levels of adrenocorticosteroids in the body and arterial pressure (Lefer 1975). One mechanism whereby these effects are exerted is the facilitatory property of corticosteroids and in particular the mineralocorticoids on the pressor effects of noradrenaline and adrenaline (Chopde *et al.* 1975, Lefer 1975). Haemorrhage has been shown in numerous studies (Gann & Egdahl 1965, Gann & Cryer 1973) to be a potent stimulus to corticotrophin and corticosteroid release. Afferent fibres from stretch receptors in the right atrium and the carotid arteries relay the information to multiple pathways in the brain that can both inhibit or stimulate corticotrophin release (Gann *et al.* 1977b). Gann & Egdahl (1965) extended previous observations to show that hypotension produced by ganglionic blockage, uncomplicated by hypovolaemia, and hypovolaemia without hypotension (achieved by concomitant administration of noradrenaline) also activated this hormonal

response. In the latter case however the possibility of nor-adrenaline itself causing the observed effect was not considered. The haemorrhage effect on the pituitary-adrenal axis is apparent within 15 minutes of the initial stimulus and the steady state of cortisol 15-30 minutes after haemorrhage is proportional to the logarithm of the magnitude of the haemorrhage.

Amongst sheep both foetal (from at least 90 days gestational age) and maternal plasma corticotrophin levels respond to haemorrhage (Alexander *et al.* 1974). Sheep in fact are more susceptible than other species examined to the pressor effects of corticotrophin (expressed as a significant increase in systolic and diastolic pressures within 24 hours) (Scoggins *et al.* 1974). The effect is not caused by corticotrophin itself or a variety of corticosteroids singly or in combination (Fan *et al.* 1975) although 17α , 20α -dihydroxyprogesterone and 17α -hydroxyprogesterone may be active in this regard (Coghlan *et al.* 1976).

EXPERIMENTAL METHODS - See §5.2

EXPERIMENTAL RESULTS AND DISCUSSION

Cannulation and Recovery

In Figure 5-15 the individual cortisol responses to the surgery, handling and adaptation to the experimental procedures are presented. The mean cortisol level during cannulation was 112.4 ± 27.5 ng ml⁻¹ (n=6). After transport to the experimental housing 4-6 hours later on the same day the mean concentration had declined to 51.9 ± 43.5 ng ml⁻¹ (n=7). Excluding sheep 258 the mean cortisol level in the day 2 samples dropped further to 18.6 ± 16.3 ng ml⁻¹ (n=10) and then to 7.4 ± 4.1 ng ml⁻¹ (n=9) in the day 3 samples. Levels subsequently showed little change excepting in sheep 258 which showed evidence of an infection with a high temperature and a poor appetite throughout the recovery period. In this animal very high cortisol release rates were apparent on several occasions (Figure 5-15b). The differences between the cortisol responses of individual sheep

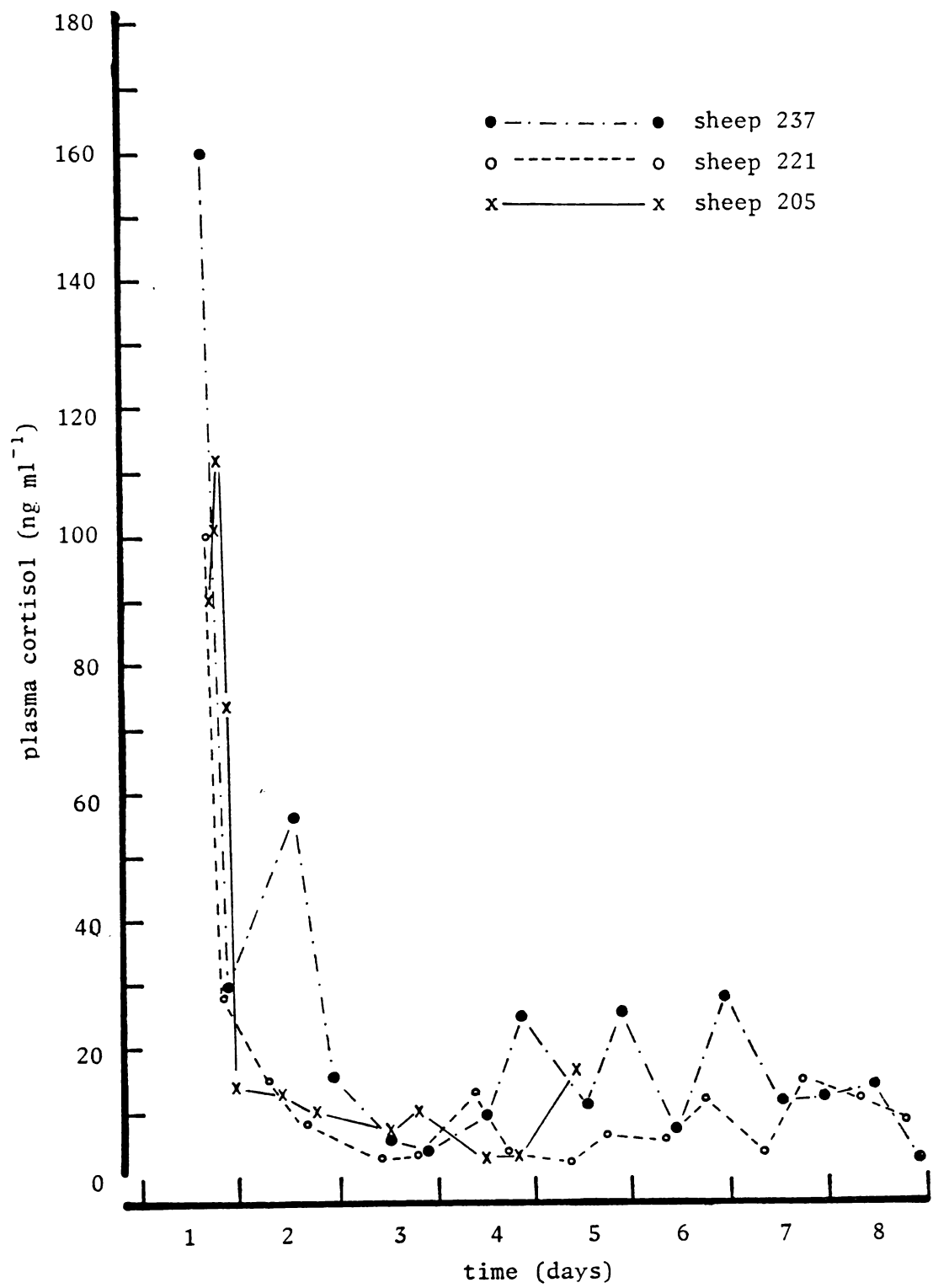


Figure 5-15a. Plasma cortisol during and shortly after cannulation (day 1) and during recovery (days 2-8). Over the recovery period samples were collected twice daily from patent cannulae.

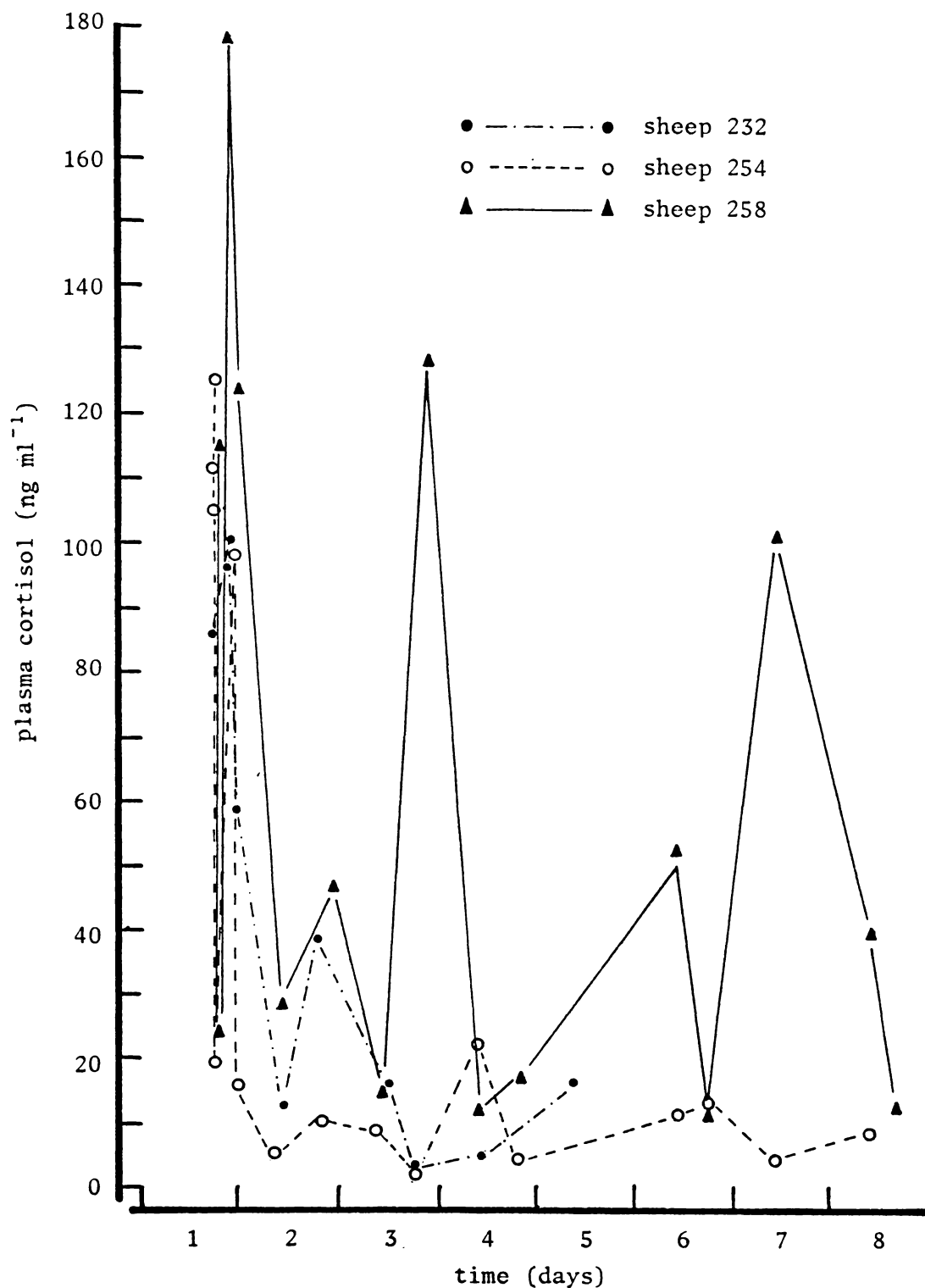


Figure 5-15b. Plasma cortisol during and shortly after cannulation (day 1) and during recovery (days 2-8). Over the recovery period samples were collected twice daily from patent cannulae.

($F_{5,44} = 6.07$) and between the cannulation and recovery phases ($F_{1,44} = 76.1$) were both statistically significant ($p < 0.001$). Omitting sheep 258 from the analysis made little difference except in slightly reducing the variation between sheep ($F_{4,36} = 4.03$, $p < 0.01$).

The concentration of cortisol found during surgery was very high, approximately 10 times that seen later in these animals during the recovery phase and at other times in this study. It seems unlikely that the superficial surgery or the halothane, alone or in combination, were responsible if the relevant data from the human can be extrapolated. It is more likely that the high level of stimulation already discussed in relation to the catecholamines (§5.3) and lactic acid (§5.4) reflects the events preceding surgery. Cortisol concentrations returned to the normal range within 48 hours.

The process of endocrine adaptation of sheep brought indoors for experimental purposes when surgical preparation is involved has not been investigated in any detail. In the present study the adaptation was initially to a new environment, food and management procedures and later to cannula implantation and recovery. Sampling stressors present where blood is collected by venipuncture, were not factors. Pearson & Mellor (1976) showed that tamed or adapted sheep and goats had significantly lower corticosteroid and glucose levels as well as a lower heart rate than animals unaccustomed to the experimental procedures employed. These authors also report that sheep and goats adjust to a new laboratory environment within 2 weeks. Sheep in the present study had at least 2 weeks to adapt to the confinement prior to cannulation and it is apparent from the cortisol values subsequent to surgery that basal levels are reached within 48 hours of the cannulation despite the very high concentrations reached during surgery.

Catecholamine Infusions

Only a limited number of the samples collected during the catecholamine infusions were saved for cortisol analysis.

l-dopa	20	$\mu\text{g kg}^{-1} \text{ min}^{-1}$	(2 sheep)
noradrenaline	1.0	$\mu\text{g kg}^{-1} \text{ min}^{-1}$	(2 sheep)
adrenaline	0.33	$\mu\text{g kg}^{-1} \text{ min}^{-1}$	(3 sheep)

L-dopa appeared to produce a modest rise in cortisol levels although in one case (sheep 237) the preinfusion plasma cortisols were already high (31.6 and 56.8 ng ml^{-1}) (Figure 5-16). Both sheep infused with noradrenaline demonstrated a large cortisol response after the end of the infusion and one (237) an infusion-associated rise as well. The infusion of adrenaline produced peaks of cortisol at varying times. In one sheep (205) there was a preinfusion rise, in another (254) a peak corresponding to the end of the infusion while in the third (237) a biphasic response with high levels during and after the infusion observed (Figure 5-16).

Several points should be made in relation to these data. First the intermittent nature of cortisol release means that significant release may have occurred between the samples taken under the blood collection regime employed. In comparison to baseline levels previously measured and the preinfusion concentrations ($<20 \text{ ng ml}^{-1}$) some significant rises in cortisol (up to 93.6 ng ml^{-1}) occurred. Enough animals were not examined however to draw any definite conclusions as to the validity of these observations but some possibilities suggest themselves in view of the foregoing review.

1. Elevated cortisol levels in 4 of the 7 infusion series correspond to the catecholamine infusions and are likely to be a direct consequence. However at the dose levels employed a cortisol response was not always evident.
2. In all 4 of the completed noradrenaline and adrenaline infusion series a postinfusion peak in cortisol occurred.

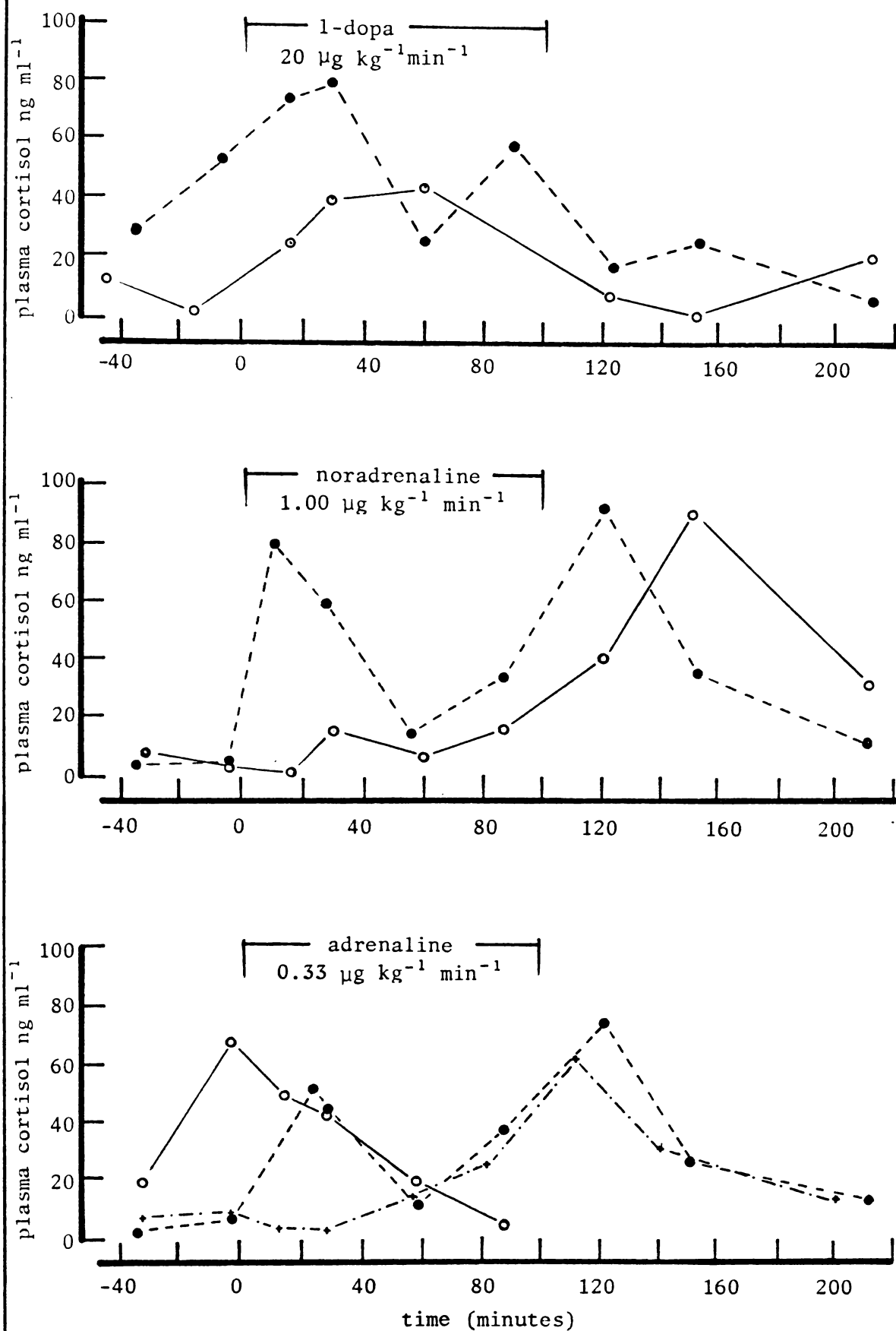


Figure 5-16. Plasma cortisol during 90 minute infusions of l-dopa, noradrenaline and adrenaline.

○ — ○ sheep 205
 ● - - ● sheep 237
 x - . - x sheep 254

In the 2 cases when this peak was preceded by an infusion peak the biphasic cortisol release dynamics discussed earlier may be invoked in explanation. The fact that cortisol levels in all 4 cases appeared to be rising before the infusion termination would support this hypothesis.

3. It seems more likely however that the very strong physiological perturbations including hypotension induced by the cessation of prolonged catecholamine infusions (§5.3) were responsible. No postinfusion peaks were observed in either l-dopa infusion. If cardiovascular effects were responsible for the post-infusion peak observed then this outcome is not surprising in view of the limited cardiovascular potency of l-dopa though since l-dopa crosses the blood-brain barrier some evidence would suggest an eventual depression of corticotrophin release by this substance (Ganong 1977).

Hypotension following prolonged adrenaline infusions in sheep is not corrected by cortisol and dexamethasone (Halmagyi *et al.* 1967). On the other hand these authors attributed the severe shock seen in sheep treated with combined α and β blockers before adrenaline infusion to an inferred suppression of corticosteroid release; a conclusion supported by the prompt relief of shock symptoms obtained with steroid administration.

4. Increased endogenous catecholamine secretion after the arrest of exogenous catecholamine infusions may be sufficient to cause or at least augment the post-infusion cortisol peak.

Thus the interesting possibility arises that the two separate peaks in the biphasic response may arise from separate phenomena, namely direct catecholamine effects and secondary cardiovascular effects. In view of the debate surrounding the control of corticotrophin release and the significance of a cortisol negative feedback in this system (Yates *et al.* 1971, Jones *et al.* 1972, Gann & Cryer 1973, Gann *et al.* 1977a) catecholamine infusions of this type may profitably be used as a model to study both adrenergic and hypotensive stimuli and the interplay between them. It is also possible that the corticosteroids may be involved in the characteristic pressure responses seen during and after long catecholamine infusions as well as modifying the metabolic consequences of such infusions (§5.4).

The data collected in the present investigation do not permit any conclusions regarding the central versus peripheral mechanism of action of the catecholamines in stimulating the pituitary secretion of corticotrophin. However the behavioural changes noted during the infusions (§5.2) indicate that the secretion of cortisol is accompanied by appropriate signs of behavioural disturbance. It is possible of course that peripheral effects such as the cardiovascular changes cause alarm and hence pituitary activation quite apart from any direct action the catecholamines might exert at the hypothalamic-pituitary level.

5.6 CONCLUSIONS

With reference to the cortisol and lactic acid levels observed in the plasma of preadapted sheep after surgical cannulation it appears that 48 hours is quite sufficient for recovery before experiments may be carried out when the perturbations envisaged are comparatively large. If small fluctuations are to be studied such as corticosteroid circadian rhythms the time required for recovery may be longer. No conclusion has been possible from this experimental design on the time taken for the catecholamines to reach basal levels after surgery.

Overall it may be said that the endocrine and physiological effects of prolonged catecholamine infusions are poorly understood. The present experiments have revealed certain relationships not previously reported in the literature but in hindsight, on the basis of pre-existing knowledge, might have been suspected. Much further work remains before the interrelationships involved can be sorted out and the cause-and-effect components identified. More sophisticated cardiovascular monitoring in conjunction with simultaneous and rapid blood sampling perhaps including samples from the adrenal vein, would do much to clarify the actual position. Tissue uptake of infused catecholamines and their subsequent release caused by the cessation of the infusion itself constitute an interesting means to further explore the various important peripheral mechanisms involved. Metabolic and other studies involving prolonged catecholamine infusions may be complicated in the short term by the postinfusion consequences and possibly also in the longer term by alterations in the ratio of the catecholamines stored in catecholaminergic structures. The disappearance of l-dopa from ovine plasma is more rapid than in man when the available pharmacokinetic data are compared with the results of the present experiment. Finally the effect of corticosteroids in modifying cardiovascular function and their release in response to adrenergic stimulation may be significant physiological mechanisms in the development of various forms of shock.

PHYSIOLOGICAL RESPONSES OF DOMESTIC STOCK TO TRANSPORTATION

CHAPTER 6

6.1 INTRODUCTION

The outcome of the physiological and social stressors resulting from the change in the environmental surroundings of transported stock has been measured in different ways, including the direct measurement of animal productivity. Whilst most stock adapt to their new surroundings and present few problems from a management point of view, instances of adverse effects range from a small depression in productive output to high mortality rates during or after transport. The causes of reduced productivity associated with transport (§6.3) have generated interest in this area of animal physiology. Behaviour patterns (§6.4) and disease incidence (§6.5) during and after transport are both affected; in the former case by the necessity to adapt to new surroundings and often a new group of animals as well, in the latter by a reduced resistance to disease. Transport has been found to influence many blood substances (§6.6) though the relationship of these substances, if any, to productivity and health is obscure in many instances.

The purpose of this chapter is to review the literature on the effects of transport on domestic animals and to relate these findings to the available information on the changes in certain blood components associated with transportation. A detailed investigation of the endocrine profile of transported lambs in the New Zealand situation is described and analysed in the context of this review and the New Zealand regulations covering

stock movement to abattoirs. Finally the concept of measuring "stress" is subjected to a critical analysis in the light of all the foregoing and guidelines for the use of blood sampling techniques are formulated.

6.2 COMPONENTS OF THE PROCESS OF TRANSPORTATION

Perhaps the major difficulty in a discussion of the effects of transport on animals is the multi-faceted nature of the process. Transport in the context of animal productivity is not simply the act of moving stock from one point to another but involves a great variety of pre- and post-transportation handling conditions, the mode of transport, environmental and social factors. All these aspects must be considered if physiological responses are to be understood. In this connection the General Adaptation Syndrome theory of Selye (1950) is useful since it permits us to predict the effects of a large number of different types of stressors. On the other hand the intensity and the "mix" of these stressors are particular to each transportation experiment and the outcome in terms of the measured parameters may in fact vary considerably between experiments. Many stressors, taken singly pose few difficulties for the adaptive mechanisms of domestic animals, but in combination with other distressing events metabolic or pathological deterioration may be rapid. The major constituents of the transportation experience of stock may be loosely classified as follows.

Environmental Climatic Conditions

Climatic conditions during transportation may be outside the range of experience and adaptability of the animals. Hyperthermia and hypothermia (both of which may occur even in moderate ambient temperatures if the air flow and ventilation are unsatisfactory), and exposure to the elements may produce severe and long-lasting effects on the stock. The time of day and the seasonal temperature may be critical. New Zealand, with a year-round temperate climate is better placed than continental

countries though clearly instances of adverse environmental exposure can still be expected on occasions.

Handling

Animals are usually subjected to intensive handling before and after transport. To animals with a history of frequent handling such as dairy cows or valuable breeding stock this may prevent few problems. With others however adverse reactions can result from the disturbance. Thus agonistic encounters, bruising and a high degree of physiological and behavioural arousal may occur even before the animals are loaded into the transporter. The presence of dogs alone is sufficient to cause significant hormonal responses in sheep - in turn initiating a cascade of metabolic events. The presence of humans may constitute a significant stressor in some instances. On arrival at the destination the whole process is repeated, this time in an unfamiliar environment.

Food and Water Intake

Lack of access to food and/or water or a diminished appetite due to other events causes weight loss and dehydration. In susceptible individuals metabolic derangement results if fasting is prolonged.

Physical Exertion

In some cases animals may be moved for considerable distances on foot and once transportation has commenced resting is limited and forced standing occurs.

Social Interactions

Domestic stock are social animals and the breakdown of social bonds, especially between a female and its young, causes considerable distress. If stock are weaned in the period immediately preceding transport then the effects of other stressors are superimposed. Mixing of unfamiliar social groups within the same pen causes restlessness and agonistic behaviour. Even the

crowding of well established social groups may cause problems between animals unable to move apart. The introduction of a new animal into an established herd or flock at the journey's end may also cause excitement, fighting and injury as the newcomer is forced to establish a place in the social grouping. While the effects on productivity are probably unfavourable the practical significance of social behavioural adaptation in this context has not been explored in detail.

Transport Type

The effect of the transport itself, with jolting, sudden starts and stops, noise, swaying and vibration may induce physical or physiological damage. Different types of transportation may have different effects. Thus while the stressor effects of air transport differ from those of ground conveyance the time spent in transit is shorter and the total effects may be less if a long distance is to be covered.

Change of Environment

The change of surroundings produces physical, physiological and behavioural responses in livestock. When animals are moved into a new environment they may encounter new foods, strange smells, new disease organisms, new stockmen and may be mixed with unfamiliar animals. They must cope simultaneously with a whole range of novel stimuli. This effect is even more traumatic when wild or feral animals are involved. .

Duration of the Journey

Transit time is one of the most critical parameters in a study of the transportation process. It may be interrupted by a number of rest periods with unloading, feeding, watering and reloading. The effects of all the aforementioned stressors though negligible or minor over short distances or elapsed time may have deleterious effects if the transit time is prolonged.

The extrapolation of data from controlled laboratory conditions to the complex process we call transport must be attempted with caution. In the subsequent sections some major interactions between physiological responses of transported livestock and the various travel components (Figure 6-1) are expanded.

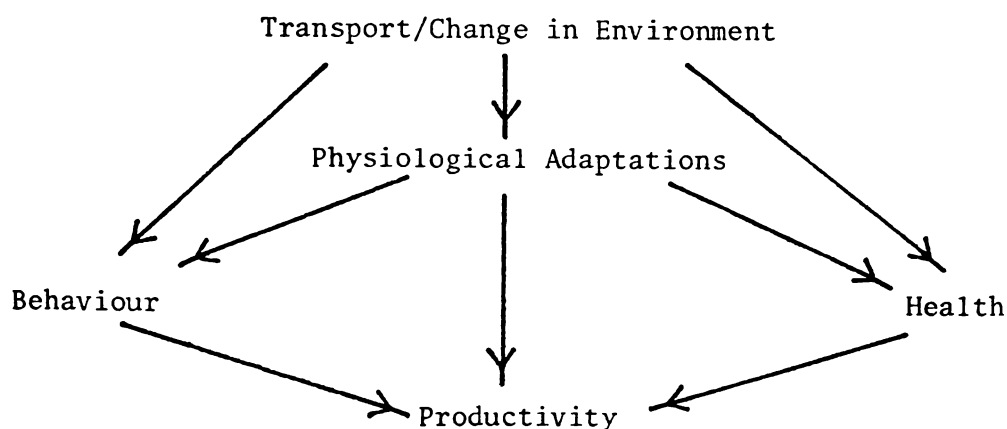


Figure 6-1. Effects of transportation on livestock

6.3 SOME CONSEQUENCES OF TRANSPORTATION WITH ECONOMIC SIGNIFICANCE

Fertility

In the ewe transport has been found to induce ovulation (Lang 1964). The effect was strongly mob-dependent and when the sheep were held in yards after transport and slaughtered in batches over 3 days the incidence of induced ovulations increased with the time of holding. When young female pigs at about the age of sexual maturity are transported and moved to a breeding herd, oestrus often occurs within a few days, apparently as a response to the stressors involved (du Mesnil du Buisson & Signoret 1962).

A depression in the fertility of heifers moved to a new herd lasting for over 12 months has been documented in a survey of non-return rates from 47 000 inseminations (Holt 1962). The

greatest reduction in fertility was found amongst animals moved within the 2 months preceding insemination (54.9% conception compared to 68.6 amongst farm-born controls) with an improving trend as time on the farm was increased. Even at 12 months the loss in fertility was 4%. Low grade genital infections to which newly introduced animals might have a low immunity were suggested as a possible cause of the adverse effects of a move to a new environment. However it is apparent from the multiplicity of physiological changes induced by transport that many other factors could be involved in the reproductive wastage, quite apart from the significant social adaptations required before integration into a herd occurs (Bremner 1975).

When semen collected from 60 bulls for 2 months following transport was compared with that collected from the same bulls in the 2 months preceding transport Willett (1957) could find no change in the conception rate in artificially-inseminated cattle in a survey of over 100 000 inseminations. The overall unaltered conception rate did however disguise significant changes in the conception rates to individual bulls. At the extremes, 13% deviations both higher and lower following the change in environment were recorded.

Bruising

An economically significant problem associated with the mustering, handling in yards, loading, transportation, unloading and yarding of slaughter stock is the physical damage resulting in bruises and other lesions to the carcass. In a minority of cases complete rejection of a badly affected carcass can occur, though usually the loss is incurred through the trimming of localized lesions and downgrading. An average figure of 0.24% condemnation rate for bruised cattle carcasses at major South African abattoirs is given by van den Heever (1961). While the outright rejections appear (given the non-uniformity in inspection standards and the limited nature of the survey) to be less frequent in Australia (Fewster 1960), the seriousness of the problem may be gauged by the fact that Hemsley (1973) quotes an estimate of a 39% incidence of

bruising in cattle carcasses assessed in a number of Victorian meatworks, 8% of which were considered serious. The average quantity of meat trimmed was 1.6 kg per head for bullocks and 2.8 kg for cows. In New Zealand it has been estimated that about 1% of the annual export beef earnings are lost through cattle bruising (Marshall 1977). Jostling, fighting and tramping among pigs cause widespread, if usually minor, damage to pig carcasses. In a survey of 9837 pigs transported to slaughter in the United Kingdom 779 or 7.9% were bruised and a further 4365 (44.4%) presented with marks, cuts and abrasions attributed to fighting (Meat and Livestock Commission 1975). Other problems listed were abscesses resulting from tail biting, faulty injection techniques, wound infection and skin blemishes from urine scald or strong disinfectants. Rough handling and poorly designed facilities exacerbate the problem (Anderson 1973, Hemsley 1973, Korn 1975). Bruising becomes much more common as the length of time in transit increases; the stock become tired and less able to avoid situations leading to damaging encounters (van den Heever 1961, Anderson 1973). Poor stockmanship may result in highly excitable, aggressive and difficult-to-handle stock with associated bruising. There is considerable scope for improvement in the management and quite handling of stock and in the design of suitable holding and transport facilities (Anderson 1973, Hemsley 1973, Korn 1975).

Weight Loss (Shrinkage)

Losses in liveweight or "shrink" during transport are a direct economic loss to the producer. Most of this weight loss is due initially to a reduction in the weight of digesta in the alimentary tract but carcass losses may also be significant if access to food is restricted for long periods. In cattle starvation for 4 days or more results in a rapid carcass weight loss (Kirton *et al.* 1972), while in lambs measurable losses are apparent after 1 day off pasture (Kirton 1973).

Long distance travel by cattle commonly results in losses of liveweight of 5-15% (van den Heever & Sutton 1967, van den Heever *et al.* 1967, Self & Gay 1972, Dow 1976). In an extensive study of 4685 feeder cattle purchased in 53 lots and shipped an average of 1027 km (range 240-1825 km) Self & Gay (1972) report an average liveweight loss of 7.7% (range for individual shipments was 4.0-12.7%). A lower loss (7.2%) was experienced when cattle were purchased from the ranch and shipped directly to the feed lot than when they were obtained via a saleyard (9.0%, $p < 0.05$). The correlation coefficient between distance shipped and % shrink was 0.72 ($p < 0.01$) within the ranch origin cattle with a regression coefficient of -0.0038% liveweight km^{-1} . The same regression coefficient was found for the saleyard cattle but here the correlation was non-significant. In a detailed study of 60 paired steers split into 2 groups, a 2.2% shrinkage was measured over a 2.75 hr (24 km) handling-transport period rising to 6% for a 1336 km (22-27 hr) journey (Self & Gay 1972). The total shrinkage of 6% over the long journey was apportioned as follows: gut contents -2.8%; intestinal tract -0.4%; eviscerated carcass -1.9%; hide -0.6%; head -0.1%; shanks -0.1%; pluck -0.2%; and liver +0.1%. Under extreme conditions (high temperatures over 4 days without access to food and water) a 19.7% reduction in liveweight was recorded in 18 cattle (van den Heever & Sutton 1967). 35.5% of the total loss was accounted for by the loss in weight of the ingesta, the digestive tract 5%, carcass weight 35.6%, blood (and some minor slaughter trimmings) 18.2%, and other viscera and organs 5.7%.

These data are confirmed with respect to the proportion of loss attributable to gut contents by van den Heever *et al.* (1967). Evacuation of intestinal contents did not exceed 5% of the initial liveweight from a total weight loss of 11.6-13.1% over a rail journey of 4-5 days. In this study it was also found that animals fed and rested *en route* in two 4 hr breaks lost only 1% less liveweight than unrested cattle. After 24 hrs watering and feeding the loss of weight at the end of the journey was reduced to between 4.6 and 8.5% over the various

experimental groups. Presumably the initial rapid weight gain experienced corresponds to a restoration of gut contents and water intake. Complete recovery from transport weight losses takes considerably longer. In the survey of Self & Gay (1972) the time taken at the feedlot to recover lost liveweight was similar for the ranch and saleyard cattle (10.4 and 10.8 days respectively). Individual shipments took between 3 and 30 days. The results of van den Heever *et al.* (1967) indicate that shrinkage is largely due to the limited access to food or water, or the complete fasting, of slaughter stock. The effect of the rail transport *per se* on the cattle liveweight was small in comparison. However Ginsberg *et al.* (1963) report that tranquilization of 40 steers over a 19 hr, 242 km journey reduced the shrinkage from 15.5 kg/head to 9.5 kg/head. The consumption of fodder by cattle *en route* tends to be depressed, well below daily maintenance requirements even when hay is offered *ad libitum* during 4 hr rest periods (Bisschop 1961). Extending the period of rest to 8 hours allowed a doubling of the food intake.

Sutton & van den Heever (1968) found a distinct downward trend in the carcass yield of Merino sheep as the rail time was increased, the effect becoming marked after 3 days and associated with other deleterious metabolic consequences. Cuthbertson & Pomeroy (1970) recorded a 0.9% loss in carcass weights comparing pigs transported for $\frac{1}{2}$ hr against those transported for 8 hrs. In a further trial with $\frac{1}{4}$ hr, $2\frac{1}{4}$ hr and 7 hr journeys, liveweight tended to decrease with increasing road time, but no significant differences were detected between carcass weights.

The weight losses by stock in transit within New Zealand have not been well documented but the distances covered are not great in most instances. Recently it has been estimated that the average distances travelled by lambs, sheep and cattle from the farm to slaughter are 121, 135 and 123 km respectively (Chudleigh *et al.* 1978).

Mastitis

An unequivocal relationship between stressors, circulating corticotrophin and corticosteroids on the one hand and cell levels in milk has not yet been established in the literature (Finn 1977). Nevertheless in several studies including corticotrophin injection (Whittlestone *et al.* 1970, Finn 1977), arousal associated with trucking of dairy cows (McFarlane *et al.* 1949, Macadam 1954, Finn 1977), and introduction into a new herd (Finn 1977) increased milk somatic cell levels and susceptibility to udder infection have been found. Increased cell counts in milk following transportation may be partly due to mechanical damage to the udder causing epithelial cell shedding and a reduced resistance to the movement of leucocytes into the milk (Macadam 1954) but probably also involves endocrine-induced adaptations including effects of corticotrophin and the corticosteroids.

Meat Quality

Ante- and postmortem factors overlap in determining the biochemical changes that influence the eventual quality of meat and for many years it has been suggested that environmental conditions prior to slaughter may effect the ultimate colour, gross morphology and organoleptic properties of postmortem muscle (Hedrick 1965). Pathological conditions including dark-cutting beef (Lawrie 1958) and pale soft exudative (PSE) pork (Hedrick 1965) are both increased by a high level of preslaughter excitement among susceptible breeds and individual animals. Pig breeds liable to sudden death caused by environmental stressors (porcine stress syndrome) are also the most likely to produce PSE pork (Nelson *et al.* 1974). The characteristics of PSE pork are a noticeably paler colour, the presence of considerable amounts of free fluid in the muscle and a very soft spongy texture. Dark-cutting beef derives its name from its purplish-red colour. The texture of dark-cutting beef may also be coarse and sticky. Although these two conditions are distinct biochemically both are related to the previous history of glycolysis, lactic acid accumulation and the rate and extent of the postmortem fall in muscle pH (Hedrick 1965, Lawrie 1966).

6.4 DISEASES ASSOCIATED WITH ANIMAL TRANSPORT

The challenge to homeostatic mechanisms posed by the processes involved in transportation results in a weakened resistance to disease. An effect of corticotrophin and corticosteroids in reducing tissue penetration by lymphoid cells (Shannon & Jones 1974) as well as weakening other immunity mechanisms (Jasani 1978) may contribute to the impairment of disease resistance. Subclinical diseases of many types will rapidly become acute particularly when long distances are covered or where especially rigorous conditions are encountered. At best the weight losses and subsequent production shortfall will be high; at worst heavy mortalities will occur. Some disease syndromes including transport fever and porcine stress syndrome are predominantly associated with transport while others, such as swine fever may be triggered during transportation (Reid 1955).

Transport (Shipping, Railroad) Fever

Transport fever in cattle is a significant problem in the North American livestock industry but also occurs elsewhere (Hails 1978). Transport fever is not a single entity but is a complex of clinical conditions caused by a number of pathogens of low virulence under normal conditions. Symptoms usually commence after the destination is reached with anorexia, depression and fever. As the disease progresses pneumonia develops (McAuliff 1956). Depressed production in the form of reduced weight gains is related to the severity of the attack (Wohler 1972) while death may ensue in up to 10% of affected mobs (Wohler 1971). The physical condition of the cattle has a direct relationship to the incidence of the disease developing after transportation. In calves so-called transit fatigue is characterized by scouring and pneumonia. Calves are particularly susceptible during the first 2 weeks of life and during cold weather (Staples & Haugse 1974).

Salmonella Infections

The growth of salmonella organisms in the rumen of cattle occurs rapidly when the diet is restricted. With access to pasture or adequate feed elimination of introduced salmonellae occurs (Brownlie & Grau 1967). Deprivation of food therefore may be a major predisposing factor for ruminant infections associated with holding in yards. In south-east Australia as many as 45% of cattle at slaughter had rumen contents contaminated by salmonellae (Grau & Brownlie 1965) while 6.6% of 1357 calves and 9.9% of 876 pigs were positive for salmonellae in the faecal or tissue samples collected at a New Zealand freezing works by Nottingham *et al.* (1972). Among New Zealand sheep the majority of isolated outbreaks are either among mobs which have travelled long distances or among mobs at freezing works (Salisbury 1958) and are associated in most instances with a high stock density, holding in yards and/or a sudden change in nutrition (Robinson 1967, Gardiner & Craig 1970). Salisbury also considered that the higher stocking rate of dairy cows as compared to beef breeds and their intensive husbandry accounts for the greater incidence of salmonellosis in dairy cows. Severe losses of sheep aboard ship have been reported where a change in diet and crowding were compounded with high ambient temperatures and high humidity (Gardiner & Craig 1970).

Transport Tetany

Hypocalcaemia sometimes in conjunction with hypomagnesaemia in domestic animals is precipitated by fasting, hot weather, exercise and excitement and is especially apt to develop in late pregnancy or early lactation. The association of transport with many of these factors had led to stock losses on occasions (Ross 1948, Sampson 1956). Transport tetany occurs in pigs and horses but ruminants are particularly at risk (Ross 1948, Green *et al.* 1953, van der Walt 1961). The incidence is claimed to be higher among beef breeds than in dairy cows. The lowered dairy cow susceptibility is possibly related to superior nutrition and adaptation to handling procedures (Ross 1948). Treatment with

intravenous calcium is only partially effective, especially once tetanic symptoms have appeared (van der Walt 1961, Pierson & Jensen 1975) indicating that hypocalcaemia may be only one symptom of a more complex aetiology.

In reindeer the decrease in serum calcium becomes greater as the length of time in captivity increases (Hyvärinen *et al.* 1976); in feedlot lambs tetany sometimes occurs within 4-5 days after arrival at the feedlot (Pierson & Jensen 1975).

Selenium-Responsive Diseases

Myopathy such as white muscle disease (muscular dystrophy) appear to be associated with either vitamin E or selenium deficiencies or both in cattle and sheep (Andrews *et al.* 1968). Abnormal conditions requiring adaptation may precipitate the disease. Donaldson (1970) describes heavy mortalities from muscular dystrophy amongst cattle being transported by sea. In this particular case low water and feed intakes were suggested as major factors. The condition, while present on the farms of origin, was clearly precipitated by on-board factors.

Ketosis (Pregnancy Toxaemia, Acetonaemia)

Hypoglycaemia, ketonaemia and high levels of plasma cortisol are clinical findings associated with ketosis (Lindner 1959, Reid 1960, Sutton & van den Heever 1968). Elevated cortisol levels and ketonaemia (§6.6) also occur during transport. Ketonaemia occurs if transport is prolonged beyond 3 days and is associated with a depletion of liver glycogen (Sutton & van den Heever 1968). Subclinical ketosis is present for transit times of less than 3 days. Transport experiments in which plasma glucose has been measured (§6.6) have generally shown an increase over the short term. Longer term experiments especially when the stock are denied access to feed might well show a decrease. The incidence of hypoglycaemia ketosis at South African abattoirs is said to be high (Sutton & van den Heever 1968).

Porcine Stress Syndrome (Malignant Hyperthermia, Fulminant Hyperthermia)

Porcine stress syndrome is a genetically-based disorder affecting modern pig breeds including the Piétrain, Landrace and Poland China developed for leanness and high muscle yield. Many of these pigs are incapable of maintaining physiochemical homeostasis when subjected to even mild stressors. By comparison the Chester White and Large White are resistant (Briskey 1964, Lister *et al.* 1970, Patterson & Allen 1971). The symptoms are tachycardia, high body temperature, severe metabolic and respiratory acidosis (caused by lactate accumulation in the blood), serum electrolyte changes with marked hyperkalaemia in the period immediately preceding death, vasoconstriction and hypertension. The massive shift from aerobic to anaerobic metabolism and the increase in body temperature eventually cause a collapse of normal physiological control and the muscles enter an irreversible rigor prior to death (Allen *et al.* 1970, Berman *et al.* 1970, Williams *et al.* 1975). Up to a 10-fold acceleration of the metabolic rate precedes the onset of clinical symptoms increasing further as the syndrome develops (Williams *et al.* 1975).

Procine stress syndrome occurs as a consequence of stressors such as anaesthesia, handling, transport, exercise, excitement and high temperature (Allen *et al.* 1970, Kallweit & Haase 1971, Allen *et al.* 1974, Smith & Allen 1976). In combination these factors have resulted in serious losses to the pig industry. Smith and Allen have demonstrated a high degree of correlation between the proportion of deaths in transported pigs and the temperature, with a seasonal peak over the summer period (Allen *et al.* 1974, Smith & Allen 1976). In continental Europe and in North America losses from porcine stress syndrome have reached serious proportions. Lendfers (1970) quotes statistics for the Netherlands showing a steady increase in such deaths from 1.49 pigs per 1000 transported in 1960 to 6.67 in 1969. Over the same period the figures for the Federal Republic of Germany are even higher (Löhr 1970) but in Britain, where the proportion of

stress-susceptible breeds is very much lower, losses are only 10% of those occurring in continental Europe (Allen *et al.* 1974).

A similar syndrome has been reported in the dog (Short & Paddleford 1973) and also in man (Britt & Kalow 1970) triggered by anaesthetics or by muscle relaxants administered during surgery. The underlying cause is unknown but excessive sympathetic nervous activity (Williams 1976), alterations in calcium distribution (Heffron & McLoughlin 1971) or of oxidative phosphorylation (Sybesma & Eikelenboom 1969), adrenal insufficiency (Marple *et al.* 1972) and abnormal thyroid hormone activity (Ludvigsen 1969) have all been advocated.

In susceptible pigs, even if death does not intervene, the meat exhibits the undesirable characteristics of pale, soft exudative pork (Nelson *et al.* 1974). The loss to the industry lies in death during transport, reduced carcass quality and reduction in saleable pork.

6.5 BEHAVIOURAL RESPONSES OF DOMESTIC STOCK TO TRANSPORT

Limited data are available on the behavioural reactions of stock in the transportation process. Yet clearly the advances in the design of facilities for stock handling and comfort have rested to a large degree on the observations of stock behaviour made by handlers. Well before conditions have become so noxious that pathological or physical damage intervenes, behavioural expression of distress will become apparent and before this less obvious species-specific changes discernible to the skilled stockman will occur. These cues may be used to identify stressful procedures and as an aid in the design of better facilities. Until recently few of these behavioural responses have been recorded in a form useful for planning.

Transported stock prefer to stand in all types of transport, although increasing numbers become recumbant as the distance of travel increases (Bisschop 1961, Sutton *et al.* 1967, Sutton &

van den Heever 1968, Kilgour & Mullord 1973, Ewbank 1975). Stock deprived of food and water for the duration of a long journey such as the oxen in the study of Sutton *et al.* (1967) or the 5 day study of transported sheep (Sutton & van den Heever 1968) have an immediate preference for water on unloading. Over unbroken intermediate distances no clear preference for food or water is apparent (Sutton & van den Heever 1968) and after comparatively shorter journeys a clear bias for food over water is evident (Bisschop 1961, Kilgour & Mullord 1973). Food intake is generally below maintenance even when the trip is interrupted by rest periods (Bisschop 1961, Sutton & van den Heever 1968, Ewbank 1975) and unless rest periods are planned with regard to natural diurnal grazing patterns food intake may be further depressed (Kilgour & Mullord 1973).

The effects of loading and unloading with the accompanying excitement may cause more behavioural disturbance than the actual transport (Ewbank 1975). This observation has not however been established in terms of physiological responses. The effects of rest periods have been claimed to be both beneficial to (Sutton *et al.* 1967, Sutton & van den Heever 1968) and without significant effect on (van den Heever *et al.* 1967) stock condition at the final destination. The disruptive effects of extra handling must be balanced against the ability of animals to rest in an unfamiliar environment. The desirability of rest periods will vary depending on the species and the transportation process in question and, most important of all, its duration. It may well be that over several days in a temperate climate, short rest periods may be of little value. Under New Zealand conditions the number and duration of rest periods needs careful investigation.

The most crucial variable on which the incidence of behavioural distress depends is the duration of transportation, although other factors will modulate the timing. This conclusion is also drawn from the physiological data reviewed in the following section. For example Kilgour & Mullord (1973) report obvious signs of fatigue and behavioural indications of distress in

trucked calves only after 13 hours in transit. The use of tranquilizers in pullets (Champion *et al.* 1966), cattle (Ginsberg *et al.* 1963) and pigs (Devloo *et al.* 1971) to reduce behavioural problems in transport has had mixed results. Problems with drug administration, duration of action, carcass residuals in slaughter stock and unpredictable reactions make the use of tranquilizers hazardous without careful investigation. In many instances other steps may be taken to reduce the causes of distress and eliminate the need for tranquilization.

No systematic study has been made of behavioural reactions in combination with physiological monitoring. Both types of data are of value, but in combination could be especially powerful. Further systematic study of the behaviour of different species under the difficult conditions imposed by stock transport is required.

6.6 PHYSIOLOGICAL RESPONSES OF DOMESTIC STOCK TO TRANSPORT

The effects of transportation on physiological variables have been measured in numerous studies. Those relating to hormones and carbohydrates are reviewed here as an introduction to the present study and as an illustration of the problems involved in studying and interpreting such a multi-factorial situation. Not only are the effects of transportation covered but also considered are the effects of other stimuli, such as mustering and fasting, that may be important elements in the experience of transported stock (§6.2).

As a result of a lack of uniformity with respect to experimental design, procedures and research models, results and conclusions are often both conflicting and confusing. The major differences between experiments include the following factors.

1. Species and breed differences make quantitative or even qualitative comparisons difficult.

2. Age-dependent responses of circulating substances are common and the age of the experimental stock differs widely.
3. The previous management experience of the animals is not usually described but this can be important particularly in the case of physiological adaptations. Extinction of some hormonal responses occurs with repeated trucking for example.
4. Weather conditions may play a major role in accentuating or damping physiological processes observed in the laboratory.
5. The form and duration of transport varies very considerably from short distances of the order of a kilometre to journeys of 5 days without access to food or water.
6. The frequency and timing of blood sampling are very important as will be shown. Furthermore the method of collecting the blood can influence the results. Together with experimental design these differences are a major source of conflicting results.
7. Analytical methods, especially some earlier procedures, contribute to the variability of the reported changes.

Thus it is often difficult to compare absolute levels of blood substances between experiments but trends in the levels of various substances may be discerned and compared.

Hormones and Transportation

The limited number of endocrine studies on transportation are virtually confined to the steroid hormones of the adrenal, beginning with Meschaks (1953), using a chemical assay procedure capable only of determining an uncertain mixture of steroids of gonadal and adrenal origin, and Holcombe (1957) with a more

specific corticosteroid assay. Improvements in assay techniques have developed to the specific binding assays for cortisol and corticosterone used in recent years. Since separate data are available relating specifically to some of the components of transport (§6.2) the hormonal responses are dissected accordingly as a guide to the interpretation of the cortisol levels found in the present experiment.

Sampling Environmental stimuli of many types increase plasma corticosteroid levels and these include blood sampling itself (Elvidge *et al.* 1976). Excitement resulting from the human-animal interaction, novelty or anticipation of the procedure and any pain caused by the sampling can be minimized if precautions are taken. The cortisol stimulus-secretion lag of several minutes (§5.5) is an advantage that may be exploited in some experimental situations; animals can also be trained or habituated to a sampling routine (Elvidge *et al.* 1976) while in other studies the animals must be cannulated if the results are not to be confounded by sampling disturbance.

In calves the effect of sampling at 10 minute intervals for 2 hours was to increase plasma 17-hydroxycorticosteroids over the 30-110 minute interval by approximately 50% from a baseline of $46.7 \pm 54.1 \text{ ng ml}^{-1}$ ($n=90$) though the effect was non-significant due to large inter-animal variation (Shaw & Nichols 1963). When samples were taken at intervals of 30 minutes or longer no effect of sampling was observed (Shaw & Nichols 1962). Willett & Erb (1972) using a more specific binding assay reported a doubling of cortisol (to $8-12 \text{ ng ml}^{-1}$) and a trebling of corticosterone (to $8-16 \text{ ng ml}^{-1}$) levels in cannulated dairy cows when the animal was aware of the sampling process. Anticipation of feeding caused rises of similar magnitude. Brief restraint with venipuncture or cannula sampling cause small rises in corticosteroid release in calves while a 10 minute restraint causes very large increases, taking over 30 minutes to restoration of normal levels (Stephens & Toner 1975).

Repeated venipuncture (at 0, 7, 15, 30, 45, 60 and 90 minutes) caused a maximum rise of 19 ± 8 ng ml⁻¹ on the first day of sampling in 4 sheep with the levels significantly higher than baseline for some 45 minutes (Pearson & Mellor 1976). Decreases in the peak size and duration occurred as the procedure was repeated weekly until it disappeared altogether after 5 weeks. This interval corresponded with the time that the sheep were judged to have become tame. The heart rate was also found to be a sensitive indicator of the reaction of the sheep to the environmental events; corticosteroid levels, heart rate and the degree of tameness were generally related (Pearson & Mellor 1976). Restraint causes large and sustained cortisol release in sheep, reaching a maximum at 30 minutes with some decline seen over the next hour (Kilgour & de Langen 1970).

Mustering Disturbance to the animal begins from the point of mustering. Two experiments where corticosteroid levels were monitored in sheep after disturbance at pasture have been reported (Reid & Mills 1962, Thurley & McNatty 1973). In both experiments rises in plasma cortisol were observed. The extent of the rise and its duration depend on the circumstances and the means used to drive the sheep (Thurley & McNatty 1973). When humans were used to move the sheep 200 yards from a field to a holding pen only small transient rises from <3 ng ml⁻¹ to 10-20 ng ml⁻¹ were found. Well trained dogs, completing the operation within 3 minutes, caused rises to 45 ng ml⁻¹ at 15 minutes while less well trained dogs caused a more rapidly attained, higher (50 ng ml⁻¹) and sustained cortisol peak. Cortisol levels had generally declined by 25 minutes where well trained dogs were employed but were still above baseline levels. With daily testing over 20 days a degree of adaptation or habituation, reflected in progressively lower cortisol peaks, was observed. After this period the response remained constant. The effect of habituation is also seen in the data of Reid & Mills (1962) where sheep accustomed to being mustered into a yard with as little excitement as possible had cortisol levels less than 13 ng ml⁻¹ (minimum assay sensitivity)

while untrained sheep usually had levels of 13 ng ml^{-1} or higher; in one case reaching 36 ng ml^{-1} .

Food and Water Deprivation Lack of food is itself a stimulus to the pituitary-adrenal axis. In sheep penned individually indoors and accustomed to *ad libitum* food intake an 18-20 hour fast caused rises in plasma cortisol from 5 to about 25 ng ml^{-1} accompanied by rises in serum free fatty acids (Purchas 1973). On the other hand if fasting was routine and periodic then an extinction in the corticosteroid response occurred. The extent of the rise was related to the daily food intake, where the greater the intake the greater the rise in cortisol at the end of fasting (Purchas 1973). The interaction of fasting and the other stressors associated with transportation have been studied by Reid & Mills (1962). In general it was found that fasting for 4 days (after the sheep had been indoors for 4 weeks) gave rises in cortisol following 1 hour of transportation of similar magnitude to those of sheep previously on pasture but greater than non-fasted indoor sheep after 1 hour of transportation. Very large increases in plasma cortisol occurred in 3 of 4 sheep brought indoors and fasted for 8 days then subjected to a 2 hour journey. Cortisol levels after transport were also correlated with the degree of undernourishment in lactating ewes during the period immediately prior to the experiment. Thus indoor housing and familiarization with humans and handling before transport appeared to reduce the cortisol response, while fasting increased it.

Change of Environment A complete change to a new environment is one of the more potent stimuli to prolonged excitation of the pituitary-adrenal axis. Kilgour & de Langen (1970) consider that isolation of sheep from their flock and subjection of the animal to a new environment are the greatest stimuli to corticosteroid release in this species. Sheep require up to 28 days to adapt hormonally to individual crating indoors after pasture grazing (McNatty *et al.* 1972, McNatty & Young 1973). Three cannulated sheep had mean cortisol levels of 33, 22, 13 and 9

ng ml⁻¹ on days 1, 7, 14 and 28 respectively, after being brought indoors (McNatty *et al.* 1972). Disruption of diurnal rhythms in plasma cortisol occurs initially but the rhythm is eventually re-established (McNatty & Young 1973). In a similar experiment utilizing 8 untamed sheep corticosteroid concentrations were on average 28±20 ng ml⁻¹ during the first week in laboratory pens (Pearson & Mellor 1976). Subsequently levels fluctuated but averaged between 16-22 ng ml⁻¹ over the next 5 weeks probably reflecting the reaction to handling and venipuncture. Calves appear to adapt quickly to a lesser change in environment (from loose boxes to close confinement in a wooden box) with a transient increase in heart rate and a 50-100% increase in plasma corticosteroids (Stephens & Toner 1975). Levels approximating baseline concentration were reached at 3 hours and remained low for the 14 days of confinement.

In an earlier study, Reid & Mills (1962) reported elevated corticosteroid levels for only 2-4 days in sheep after being moved indoors, increased by fasting either before or after the shift. A 7 minute transportation period and an overnight stay in an unfamiliar environment increased the plasma cortisol levels of sheep from about 10 to 30 ng ml⁻¹ (Purchas 1973).

It is clear from these studies that changes in the environment of sheep, particularly when small groups are isolated indoors will cause an increase in adrenal corticosteroid output gradually declining as the animals become habituated. The response varies considerably between animals and levels tend to be erratic and easily influenced by secondary stressors and novel stimuli in the new surroundings.

Short Term Transportation Studies The effect of transportation lasting a few hours or less under experimental conditions is to markedly increase plasma cortisol. Kilgour & de Langen (1970) estimate a cortisol level of 67 ng ml⁻¹ in 12 sheep after a 90 minute journey by truck. This compares with a level of 36 ng ml⁻¹ in blood obtained under conditions of minimal disturbance. Given

baseline levels reported subsequently, and in the present study, 36 ng ml^{-1} is comparatively high but probably reflects improved specificity of the binding assay compared with the fluorimetric assay of de Langen & Whittlestone (1970). Nevertheless these figures may be compared with levels after slaughter by throat cut of 111 ng ml^{-1} and 113 ng ml^{-1} after shearing lasting 10-15 minutes (Kilgour & de Langen 1970).

Shearing, slaughter or a 7 minute journey all caused comparable increases in ovine cortisol concentrations (to $35\text{-}45 \text{ ng ml}^{-1}$) in the study of Purchas (1973). It is of interest that a second journey of 7 minutes after an overnight stay in a strange housing facility caused a similar absolute rise in cortisol but from a much increased pretransport level - indicating an additive property of different stressors on sheep cortisol concentration. Stephens & Toner (1975) measuring $17\text{-}\alpha$ -hydroxycorticosteroids and corticosterone found increases in plasma levels of calves generally peaking at 30 minutes in a journey of 1 hour, remaining high at the cessation of transport and falling, though still not to baseline, at 1 hour postjourney. Confinement of the calves in cubicles on the truck seemed to reduce the peak level compared to unconfined transport. Reid & Mills (1962) found the effect of a 1 hour trip on 4 sheep separated from a larger mob was to increase cortisol from $<13 \text{ ng ml}^{-1}$ to between 20 and 60 ng ml^{-1} . Values had fallen substantially at 2 hours following the end of the journey but were still above 13 ng ml^{-1} at 4 hours. In a further experiment in which samples were collected during a 4 hour journey, levels showed a marked decline at 4 hours in the 2 ewes with the greatest initial cortisol response at 2 hours.

Long Term Transportation Studies When the available data on corticosteroid response to transport under commercial conditions are examined the picture is less clearcut and it is apparent that both increased and decreased levels occur. In the early study of Meschaks (1953) levels of "neutral steroids" in the urine of 6 bulls were in the normal range after a 20-40 km journey but after 1 month at the new site were elevated. In 2 Swedish

Highland heifers moved about 300 km an increase in secretion of "reducing steroids" at 1 month was followed by a decrease below the level measured on arrival and then recovery to this level after 3 months (Holcombe 1957). Similar patterns were observed in bulls though marked inter-animal variation was present. Both decreased and increased levels of 17-hydroxycorticosteroids were induced in calves by shipping from Montana to Wisconsin but thereafter levels remained low during the first week rising to elevated levels at 3 weeks and becoming normal at 1 month (Shaw & Nichols 1964). When preweaned and preconditioned (to the feed and associated facilities) beef calves are compared to calves weaned the day before transport, the preconditioned group have significantly lower corticosteroid responses prior to and during transport (Wieringa *et al.* 1976). On the other hand corticosteroid concentrations in the unweaned calves fell significantly below levels in the preconditioned group on arrival (Wieringa *et al.* 1976).

The phenomenon of reduced corticosteroid levels can be explained by exhaustion of adrenal stores or pituitary corticotrophin, feedback inhibition of corticotrophin release, or increased metabolic utilization of corticosteroids. While all of these arguments have been advanced there are very few data available making a choice possible between them under the conditions of animal transport.

Overall the data in the area of hormonal responses of domestic stock to transport are incomplete, fragmentary and confined to the corticosteroids. The following conclusions may however be drawn.

1. Unless precautions are taken sampling will cause increments in plasma corticosteroids. Only with cannulated animals from which the sample is taken very rapidly or without the animal being aware of the procedure can levels of corticosteroids be said to be a true baseline. Tame animals may give results

approximating baseline levels but they may react differently to experimental procedures from untamed conspecifics. Rapid changes in corticosteroid levels occur, with increases generally faster than decreases necessitating frequent sampling. Few studies in the transportation field have taken sampling disturbance into account.

2. Mustering increases corticosteroid levels to a moderate degree, depending on the nature of the handling and the degree of habituation.
3. Starvation increases the corticosteroid response to other stressors.
4. Different concurrent stimuli tend to cause an augmented plasma corticosteroid level, though more data are needed to confirm the nature of the additive effect.
5. Short transportation periods cause rises in plasma cortisol of moderate to high levels, somewhat less than in slaughter or shearing.
6. Long transportation periods and a new environment cause complex changes in corticosteroid levels in both directions that may extend over some months.

Carbohydrates and Transportation

Rises in blood glucose concentrations are generally associated with the capture of wild (Franzmann & Thorne 1970) or semi-wild (Hyvärinen *et al.* 1976) stock, and the handling, droving or transport of domestic animals (Reid & Mills 1962, van den Heever *et al.* 1967, Sutton & van den Heever 1968, Tollersrud *et al.* 1971). The level of blood glucose in transported animals is primarily dependent, in the initial stages, on the degree of sympathetic stimulation and, if the journey is prolonged, on muscle and liver glycogen reserves.

Effects of the Catecholamines Injected and endogenous adrenaline stimulates hepatic glycogenolysis causing a sustained rise in blood glucose levels (§5.4). In short term transport experiments the level of glucose may continue to rise after the journey is completed in contrast to the falling level of cortisol (Reid & Mills 1962). In exercise adrenaline may also have the effect of increasing the rate of muscle glucose utilization acting to negate the sympathetically-induced hyperglycaemia (Dill *et al.* 1935). Increased utilization of energy substrates may account for the lack of a significant change in glucose seen in mustered sheep (Reid & Mills 1962) and corralled reindeer (Hyvärinen *et al.* 1976) although increases in lactic acid were observed in both instances. Movement of sheep to a new environment indoors is sufficient to elevate glucose levels for 24-28 hours, although the level did not exceed 55 mg dl^{-1} after 8 hours compared to control values generally between $20\text{-}40 \text{ mg dl}^{-1}$ (Reid & Mills 1962). It should also be noted that 2 of the 12 sheep involved in this experiment exhibited marked hypoglycaemia. The stressor effects of droving and motor transport on the hyperglycaemic responses of lambs previously housed indoors is markedly greater than in pasture-grazed lambs of the same age (Tollersrud *et al.* 1971). The modification of sympathetic responses to any given stimulation by the previous experience of an animal (McCarty & Kopin 1978) may also be reflected in the lactic acid response (Plaut *et al.* 1975).

Availability of Glycogen Reserves The availability of liver glycogen reserves may alter the response to transportation. Liver glycogen reserves are dependent on the rates of muscle glycogenolysis and hepatic gluconeogenesis both of which processes are accelerated by adrenaline (Himms-Hagen 1967). Where transport times are of 2 or more days glycogen depletion (due to increased energy substrate utilization and reduced intake) and subclinical ketosis may result (Sutton & van den Heever 1968). A drop of 25.6% and 72.8% in liver glycogen in Merino sheep after 3 and 4-5 days *en route* was recorded. However glycaemic responses in animals fasted for periods of 4 days prior to a short

journey (Reid & Mills 1962) or for 4 days during transport (van den Heever *et al.* 1967) may in fact be greater than in corresponding fed controls, presumably due in part to greater sympathetic arousal. Long term hyperglycaemia is also seen in wild sheep after capture and captivity for 12 days (Franzmann & Thorne 1970). With even longer periods of fasting such as 8 days when liver glycogen depletion is correspondingly greater a depressed level of blood sugar in response to transport may be found (Reid & Mills 1962). In general the glycaemic response is directly related to bodily condition (Reid & Mills 1962).

6.7 PHYSIOLOGICAL RESPONSES OF WEANED LAMBS BETWEEN THE FARM AND SLAUGHTER

While there are in excess of 50 published studies of the biochemical changes in the blood of transported stock, the complete pre-slaughter handling period has not been systematically studied. Humane considerations for the welfare of stock to be slaughtered have concentrated on electrical stunning and blood letting but should in fact include the complete farm-to-slaughter process (Kilgour 1978a). Such a study would encompass the period before the animals leave the farm and proceed during transport to the abattoir, include washing and general handling until the animal is restrained, stunned and slaughtered.

In the investigation described blood samples for the determination of noradrenaline, adrenaline, cortisol and lactic acid were collected from 7 cannulated Dorset Horn lambs on 15 occasions beginning on the farm and continuing over 30 hours until slaughter. Nineteen other non-cannulated members of the flock were also sampled at the beginning of the study and at slaughter. The experiment was designed in such a way that each segment of the farm-to-slaughter process (including a 160 km journey) reproduced as closely as possible the experience of lambs sent to slaughter under New Zealand conditions.

EXPERIMENTAL METHODS

The experimental animals were 26, 6-7½ month old weaned Dorset Horn wether lambs, which had been subjected to normal on-farm handling. On day 1 they were shorn around the neck to permit easy access to the jugular vein and the carotid artery and 9 lambs were drafted from the mob for carotid artery cannulation on days 2 and 3.

Carotid Cannulation The surgical procedure for the cannulation of the carotid artery was as described in §5.2. The distal end of the cannula was taken under the skin from the incision site and brought out onto the dorsal surface of the neck, taped to a horn and occluded with a 16 gauge, sealed, blunted syringe needle.

Recovery After confinement indoors overnight the animals were freed to graze for 2 or 3 days (depending on the day of surgery) prior to the experiment on days 6 and 7. Thus the recovery period varied between 60 and 84 hours. The data of Chapter 5 indicate that 48 hours is a sufficient time to permit restoration of hormonal plasma concentrations to baseline levels. During the recovery period the lambs were quietly gathered in a race twice daily and the cannulae flushed with heparinized saline (500 IU ml⁻¹). On the morning of the transportation (day 6) 7 of the sheep had retained patent cannulae.

Blood Collection Fifteen millilitre blood samples could be easily and quickly obtained by removing the stopper and allowing the blood to flow under arterial pressure into a numbered test tube. The cannulae were backflushed with heparinized saline (50 IU ml⁻¹) before resealing. With the cannula taped to the horn and the close packing of the lambs, restraint was seldom necessary. Blood was collected from the cannulated sheep in a random order at each sampling to prevent any time-dependent bias caused by the sampling process on hormone levels from individual lambs.

Sampling Scheme Over the course of a 30 hour progress from the farm to slaughter 15 different blood samples were collected from each cannulated lamb (Figure 6-2). Beginning at 7.30 am on day 6 the lambs were quietly gathered into a race, without the use of dogs, and blood samples were collected from the cannulated lambs. Sampling generally took from 10 to 15 minutes. Samples were also collected from each of the non-cannulated animals by venipuncture - with handling and restraint kept to a minimum. The flock was vigorously yarded through several paddocks for 15 minutes with 2 dogs to exert maximum pressure and a sample was collected from the cannulated lambs before loading the mob onto an open-trayed truck at 10.15 am. No food or water was available from this point onwards to slaughter. After standing the lambs in the stationary truck for 15 minutes a third sample set was obtained. The truck then made two 66 km circuits over winding hill country roads representative of much of the New Zealand terrain. The advantage of a horned breed of sheep in sample collection proved to some extent a disadvantage during transportation as cannulae were readily hooked by the horns of adjacent lambs. One cannula was dislodged in this manner, reducing the number of subsequent blood samples in each set to 6. Samples were collected on the stationary truck at the end of each circuit before a 60 minute stop representing the lunch break and a final 28 km journey to the abattoir arriving at 3.15 pm. Another sample set was obtained on arrival. The total elapsed time on the truck was 5 hours. At the abattoir the flock was subjected to normal handling procedures. After unloading they were belly-washed by a water spray from below and then forced to swim a 33 m long water bath. Sampling was carried out immediately before the water bath, 10 minutes afterwards when the lambs had been penned in covered yards and again another 40 minutes later. Three further collections were made overnight at 7.45 pm, 1.15 am and 5.50 am. The next sample was at midmorning when the flock had been exposed to the noise and activity of dogs and other sheep mobs passing down adjacent races to slaughter. The penultimate sample was taken after the midday lunch break when the lambs had been drafted to the

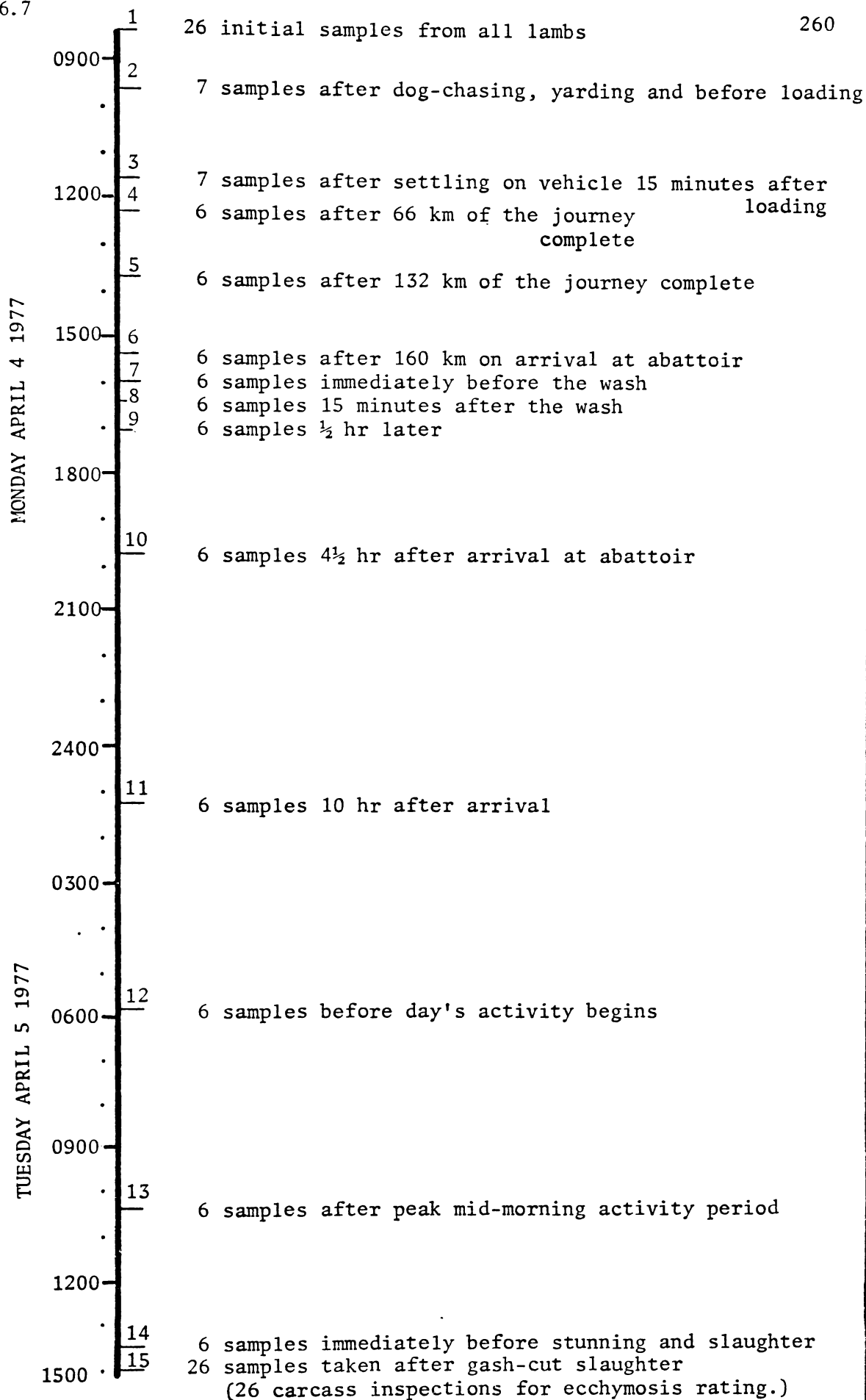


Figure 6-2. The schedule of blood sampling during the transport experiment.

base of the restraining conveyor by dogs and men. The lambs were stunned with a Thornton Mark I electrical stunner (Thornton Engineering, Auckland, New Zealand) (100 V, 0.75 amp, 50 Hz applied to the head for 1-2 seconds). A final gash-cut blood sample was obtained from all 26 animals at slaughter and the carcasses were scored for blood splash (ecchymosis) (§7.4). The full co-operation of the Auckland Farmers' Freezing Co-operative Ltd (Horotiu) employees was given at all times and apart from minor delays of the order of 10 minutes for sampling before the water bath and before slaughter the lambs were treated according to normal practice.

Sample Analysis The 15 ml blood samples were chilled and split into 2 portions (one of 8-9 ml for catecholamine analysis (§4.3) and the other of 6-7 ml for lactic acid (§4.7) and cortisol (Appendix III) analyses).

Statistical Analysis A least squares analysis of variance of the assay data from the 6 cannulated lambs was carried out to test for the significance of the effects of sample set, animal and the first sample collected in each set versus the remainder.. Sample set 15 was excluded from this analysis because of the abnormally high catecholamine levels caused by the electrical stunning. The analysis employed the logarithmically transformed data of the cortisol and lactic acid results and also the logarithmically transformed noradrenaline and adrenaline data, after 1.0 was added to each value because of the existence of zero concentrations in the catecholamine data sets. Tests for significant differences between sample set means were made using the t tables of Games (1977).

RESULTS

Weather

The weather conditions over the 2 days were partly cloudy, humid and warm with little wind (Table 6-1).

Table 6-1

Ruakura Weather Conditions During Transport Experiment

	Humidity (%)	Maximum (°)	Minimum (°)	Sun (hours)	Rain (mm)
April 4 1977	96	21.3	5.8	3.2	0.0
April 5 1977	97	24.3	9.1	5.9	1.2

Behaviour

During the period of travel the lambs slowly changed their position about the truck deck. As with calves (Kilgour & Mullord 1973) lambs tried to avoid being in contact with the outer rails of the sheep crate. The riding up on the backs of the group by some lambs was most prevalent at the start of movement after the truck had stopped for some purpose, though overall the lambs travelled more quietly as the journey proceeded. Two lambs lay down at times during the journey though they always got up during especially winding parts of the route when the animals were buffeted about. Even with normal tight packing of lambs, buffeting is a factor and some movement of animals within the group is experienced. The lambs appeared to become accustomed rapidly to the presence of the sampler and no behavioural signs of distress could be discerned. One lamb (M720) however had to be restrained and its head held high for the blood to be collected, since the cannula was blocked or kinked in its normal position. The restraint was reflected in the catecholamine levels of the blood samples taken from this lamb.

Assay Results

The overall assay results are presented in Figure 6-3 and the statistical results in Tables 6-2 and 6-3.

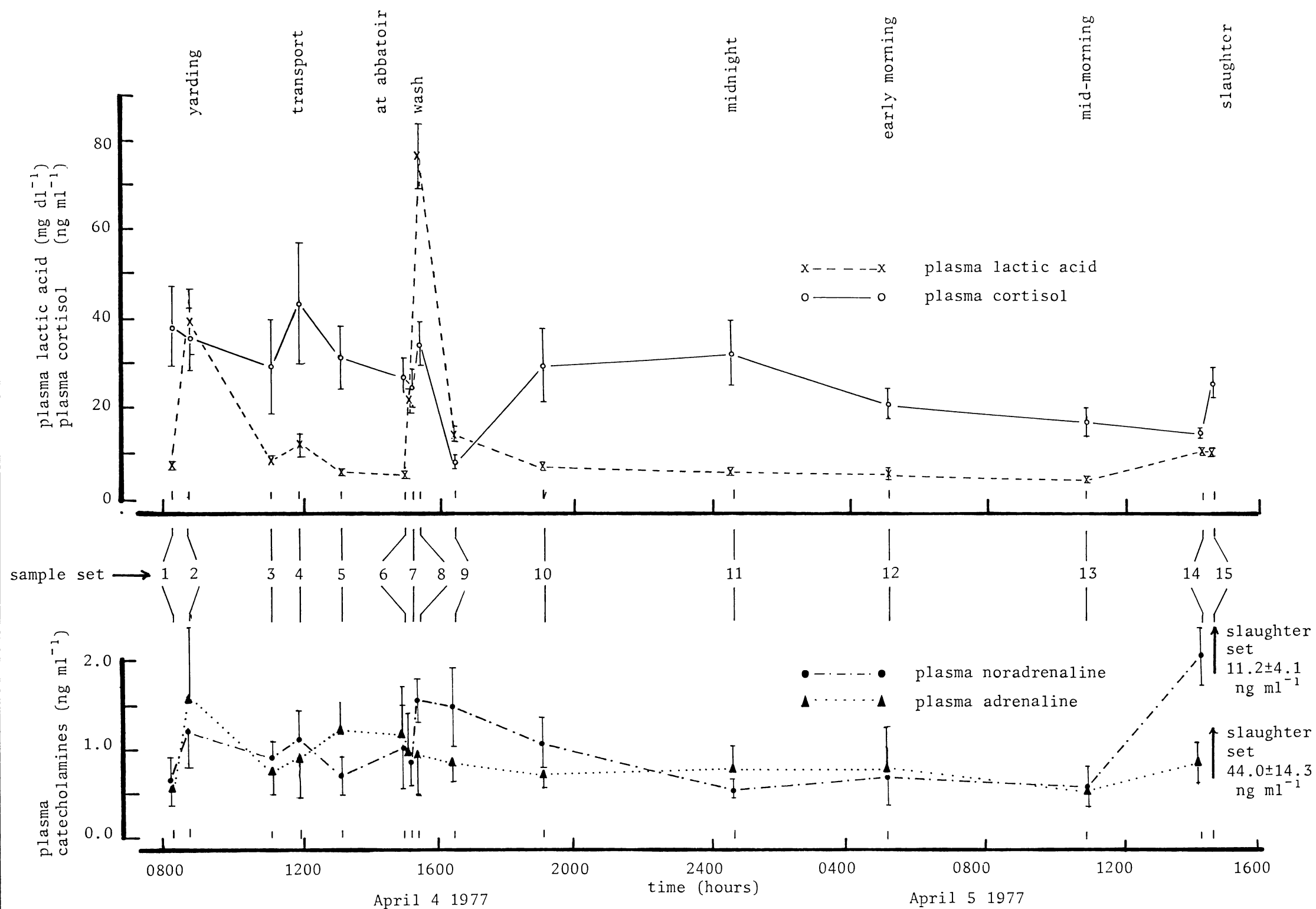


Figure 6-3. Lactic acid, cortisol and catecholamine profiles of 6 lambs over yarding, transport (160 km), abattoir handling and slaughter. Group means $\pm$ SE.

Table 6-2

Summary of the Results of the Analysis of Variance

log lactic acid					
sample set	$F_{13,64}$	=	46.575	$p < 0.001$	***
animal	$F_{5,64}$	=	1.643	$p = 0.161$	ns
sample order	$F_{1,64}$	=	0.039	$p = 0.845$	ns
log cortisol					
sample set	$F_{13,64}$	=	5.626	$p < 0.001$	***
animal	$F_{5,64}$	=	5.711	$p < 0.001$	***
sample order	$F_{1,64}$	=	9.771	$p = 0.003$	**
log (noradrenaline + 1.0)					
sample set	$F_{13,63}$	=	3.180	$p = 0.001$	***
animal	$F_{5,63}$	=	11.966	$p < 0.001$	***
sample order	$F_{1,63}$	=	0.061	$p = 0.806$	ns
log (adrenaline + 1.0)					
sample set	$F_{13,63}$	=	1.282	$p = 0.248$	ns
animal	$F_{5,63}$	=	31.678	$p < 0.001$	***
sample order	$F_{1,63}$	=	0.437	$p = 0.511$	ns

ns = non-significant

** $p < 0.01$ *** $p < 0.001$

Table 6-3

Significance of the Difference Between Consecutive Sample Sets¹
and Between Each Sample Set and the Lowest Mean Sample Set²
(logarithmically transformed data)

Sample Set	Lactic Acid	Cortisol	Noradrenaline	Adrenaline
1	(*)	(***)		
2	*** (***)	(***)		
3	*** (**)	(***)		
4	(***)	(***)		
5	**	(***)		(+)
6		(***)		
7	*** (***)	(***)		
8	*** (***)	(***)	(+)	
9	*** (***)	*** (-)	(+)	
10	***	*** (***)		
11		(***)		
12		(**)		
13	(-)	(+)	(-)	(-)
14	*** (***)	(+)	*** (***)	

¹ statistical significance of differences between consecutive sample sets indicated without parentheses

² statistical significance of differences between lowest sample set and all others indicated in round parentheses

(-) lowest mean sample set

+ p<0.1 * p<0.05 ** p<0.01 *** p<0.001

Lactic Acid Lactic acid levels in the plasma of the cannulated sheep were low at the initial yarding ($7.48 \pm 1.57 \text{ mg dl}^{-1}$) though still about twice the baseline levels observed in the ewes of the previous work (§5.4). In contrast lactic acid levels rose significantly ($39.22 \pm 17.29 \text{ mg dl}^{-1}$) ($p < 0.001$) after vigorous yarding with dogs. This response was undoubtedly the result of the exercise combined with sympathetic nervous discharge. The relative importance of these two factors in the perturbation of plasma lactic acid over the course of the experiment is not possible to estimate, but in this case anaerobic oxidation would be an important contributor. The concentration had fallen significantly ($p < 0.001$) after the lambs had been on the stationary truck for 15 minutes, rising (non-significantly) to a mean of $12.28 \pm 6.85 \text{ mg dl}^{-1}$ after the first 66 km leg of the journey. At the conclusion of the second leg of the journey these levels had receded ($p < 0.01$) to $5.80 \pm 1.29 \text{ mg dl}^{-1}$. Lactic acid, cortisol and noradrenaline all tended to fall over the second half of the transportation (Figure 6-3) suggesting some adaptation to the trucking conditions and confirming the subjective observations of changes in the behavioural responses of the stock. The initial rise in lactic acid during transport may have been adrenergic in origin since the muscular exertion needed to remain upright on the truck would be a constant factor throughout the journey. A significant increase in lactic acid ($p < 0.001$) between samples 6 and 7 (from unloading to after the belly wash) was followed by a very large ($p < 0.001$) increase to $76.27 \pm 17.89 \text{ mg dl}^{-1}$ after the water bath. The considerable effort required to swim the length of the water bath would be expected to result in very high lactic acid level but sympathetic arousal might have also been important at this time. By 45 minutes postwater bath the plasma concentration had decreased to $14.85 \pm 3.90 \text{ mg dl}^{-1}$ ($p < 0.001$) then fell further to a level approximately baseline ($6.95 \pm 1.26 \text{ mg dl}^{-1}$) at 7.45 pm. The nadir for the experiment was achieved at 10.25 the following morning ($4.47 \pm 1.24 \text{ mg dl}^{-1}$). With the disturbance and mustering of the mob up to the foot of the slaughter chain lactic acid levels again rose significantly ($p < 0.001$); here adrenergic activation seems likely to have been a major cause.

No further change was seen at slaughter. Unlike the catecholamines neither cortisol nor lactic acid have time to rise in the plasma in response to electrical stunning and slaughter in the 10-30 seconds after stunning that it takes to collect the samples (§7.3).

Cortisol Cortisol in the plasma of the cannulated lambs from the time of the first sample until they were penned after washing at the abattoir remained high, with no significant differences between sets (Figure 6-3). The cortisol concentration of the initial sample ($38.02 \pm 22.58 \text{ ng ml}^{-1}$) was higher (as were the catecholamines) than anticipated in view of the behavioural observations and the effort taken to keep disturbance to a minimum. It is possible that a significant component of the cortisol content of the initial sample sets may be traced to the peak diurnal release of cortisol which occurs at about this time of the day (McNatty *et al.* 1972). The possibility that initial cortisol levels were high because of the surgical implantation of cannulae $2\frac{1}{2}$ - $3\frac{1}{2}$ days previously is unlikely, in view of the recovery curves seen in the previously examined sheep (§5.5). Furthermore additional stimuli to cortisol release during yarding and transport would be expected to raise levels above that initially observed. In fact this did not occur (Figure 6-3). Nevertheless these lambs may have had too little time to adjust to humans and to the cannula flushing procedure of the previous 2-3 days.

Yarding with dogs did not produce a change in cortisol concentration. Thus under these conditions where the mob was maintained intact mustering may be classed as only moderately arousing, even when dogs are used. A decline in the cortisol level was found after the sheep had been standing on the truck for 15 minutes, followed by a rise during transport at the 66 km stage and falls at 132 and 160 km. All these changes were however not statistically significant. Unlike lactic acid little change was associated with either belly or immersion washing. Over the initial half of the experiment the mean cortisol levels remained

in a comparatively narrow band of 25-44 ng ml⁻¹ indicating a continuous but not excessive pressure of environmental stressors.

The most striking feature of the cortisol responses over the experiment was the marked fall ($p < 0.001$) to near baseline levels (8.12 ± 3.07 ng ml⁻¹) 50 minutes after the water bath and after 40 minutes in a covered pen. In fact this sample set differs significantly from all others at the 0.1% level - excepting sets 13 and 14 where the difference still approached significance ($p < 0.1$). The drop in cortisol secretion was promptly followed by a rise ($p < 0.001$) overnight during the quietest period of the experiment, reaching a maximum (32.60 ± 17.65 ng ml⁻¹) at 1.15 am followed by a decline in subsequent samples until the period immediately prior to slaughter. In undisturbed sheep adapted to an indoor environment cortisol release is very low between 8 pm and 8 am (McNatty *et al.* 1972).

Noradrenaline The analysis of variance amongst the noradrenaline levels showed that a highly significant difference existed between sample sets ($p < 0.001$), between animals ($p < 0.001$) but not between the first sample of each group compared to the remaining 5 ($p = 0.806$). Excluding the final set of samples at slaughter (these form a separate group discussed separately) the mean noradrenaline concentration measured over all samples for both cannulated and non-cannulated sheep was 1.1 ± 1.1 ng ml⁻¹ ($n = 105$). This is 3-4 times that found in resting subjects (§5.2) and indicates a moderate degree of sympathetic activation through the transport and handling processes. The overall mean does however disguise variations in the level of noradrenaline at various stages and also includes a disproportionate contribution by lamb M720 (Table 6-5). After yarding (sample set 2) the mean noradrenaline concentration had not altered significantly from the first sample and in fact did not change significantly between consecutive sample sets until towards the end of the experiment (sample set 14). The mean noradrenaline secretion showed a decline over the transport period and then a rise to 1.52 ± 1.16 ng ml⁻¹ after the water bath but fell slowly over each subsequent

sample to reach the experimental minimum ($0.57 \pm 0.59 \text{ ng ml}^{-1}$) at 10.25 am. This level was less ($p < 0.1$) than in the two wash samples and was significantly lower ($p < 0.001$) than the next sample taken at the foot of the slaughter chain ($2.03 \pm 0.83 \text{ ng ml}^{-1}$). In humans circulating levels of the catecholamines and cortisol are lower at night than during the day (Turton & Deegan 1974). The noradrenaline found in the blood samples collected from the gash cut wound of all sheep rose very sharply to $11.7 \pm 13.1 \text{ ng ml}^{-1}$ ($n=26$). This concentration, 6 times higher on average than the previous sample set, is a direct consequence of the massive sympathetic stimulation caused by the electrical stunning and discussed in detail in Chapter 7. As has been found on other occasions the range of values was very large (0.3 to 62.4 ng ml^{-1}).

Adrenaline The overall experiment concentration of adrenaline (excluding sample set 15) was $1.1 \pm 2.1 \text{ ng ml}^{-1}$ ($n=105$) and, like the noradrenaline results, indicates a moderate degree of adrenergic activation overall. Significant changes in the rate of adrenaline release over the course of the experiment were not revealed by the sample group means ($p=0.248$). A small rise was observed at the second sampling on the truck but levels remained about 0.5 - 1.0 ng ml^{-1} at most times. If the sharp drop in cortisol after the water wash is excluded then all 4 measured compounds reached their nadir at 10.25 am on the day of slaughter after overnight rest. No preslaughter rise in adrenaline was detected but levels jumped to a mean of $55.2 \pm 33.3 \text{ ng ml}^{-1}$ ($n=26$) after electrical stunning. Significant differences ($p < 0.001$) were observed between animals in their adrenaline responses over the experiment. But, as for noradrenaline, the disproportionate contribution to the sample means by lamb M720 was largely responsible (Table 6-5).

Comparison of the Cannulated Lambs with the Non-Cannulated Lambs

Two blood samples were obtained from all the non-cannulated lambs, one at the start of the experiment (sample set 1) and the other at slaughter (sample set 15). The samples collected from the two groups of lambs are strictly comparable in the latter case since the collection conditions were identical. The initial sample collections however differ in several important respects. First, the samples were collected from the venous circulation of the uncannulated lambs but from the arterial circulation of those cannulated. Significant arterio-venous concentration gradients have been reported for lactic acid (Andres *et al.* 1956) and for the catecholamines (§5.2). Second, the non-cannulated lambs were caught, forcibly restrained and positioned for venipuncture. The overall sample means between the two groups (Table 6-4) exhibit some differences in the first sample set but homogenous sample populations in the slaughter set.

Table 6-4

Levels of Blood Substances Measured in Cannulated and Non-Cannulated Lambs

	n	Lactic Acid (mg dl ⁻¹)	Cortisol (ng ml ⁻¹)	Nor-adrenaline (ng ml ⁻¹)	Adrenaline (ng ml ⁻¹)
<u>Sample set 1</u>					
Cannulated	7	8.21± 2.41	39.43±20.95	0.65± 0.55	0.73± 0.43
Non-cannulated ¹	19	44.72±18.16	27.52±17.47	1.50± 1.69	1.97± 4.55
Significance ²		p<0.001	ns	ns	ns
<u>Sample set 15</u>					
Cannulated	7	11.47± 2.18	25.89± 8.07	10.29± 9.49	39.90±33.85
Non-cannulated ¹	19	13.32± 3.94	22.41±10.28	12.28±14.36	60.79±32.15
Significance ²		ns	ns	ns	ns

¹ samples collected by venipuncture

² 2-tailed t test using logarithmically transformed data with allowance for non-identity of sample variance where appropriate

The highly significant increase in the lactic acid levels of the non-cannulated sheep compared to the cannulated sheep in sample set 1 was presumably a direct result of the excitement and struggle associated with restraint but also because venous lactic acid levels are higher than arterial levels (Andres *et al.* 1956). The mean cortisol concentration of the non-cannulated sheep (27.52 ± 17.47 ng ml⁻¹) compared to 39.43 ± 20.95 ng ml⁻¹ in the cannulated sheep at the start of the experiment tended towards a significant difference ($p < 0.1$) by the median test, but no difference by the same test was evident in the slaughter samples. The cortisol response of the lambs subjected to venipuncture would not have had sufficient time to reflect fully the restraint stress imposed (§5.5). While the catecholamine values were not significantly different either by the *t* test or the Mann-Whitney test, they were higher on average amongst the non-cannulated lambs (even when the exceptionally high contribution by lamb M823 is excluded). This result is in line with expectations and occurred despite the existence of a negative arterio-venous gradient in blood catecholamine content (§5.2). No significant correlation was found between the initial and the slaughter concentrations of any of the 4 measured substances in individual sheep.

Variations Between Lambs

The animal subclass means for the first 14 sample sets showed significant differences between animals in cortisol, noradrenaline and adrenaline (Table 6-5).

The differences amongst the cortisol means cannot be attributed to any specific event or combination of events but presumably are the result of individual pituitary-adrenal sensitivity to the transportation process, conditioned by events occurring in the previous history of these lambs. Most of the variance in the noradrenaline (77%) and adrenaline (85%) values between animals may be traced to a single lamb (M720), where difficulties in sampling necessitated restraint resulting in mean levels of

Table 6-5
Mean Individual Values for Blood Substances
in Cannulated Lambs

Lamb	Lactic Acid (mg dl ⁻¹)	Cortisol (ng ml ⁻¹)	Noradrenaline (ng ml ⁻¹)	Adrenaline (ng ml ⁻¹)
M720	17.14±17.28	30.14±14.02	2.15±0.77	2.60±1.30
M727	14.63±15.86	17.01± 8.41	0.61±0.53	0.55±0.26
M871	15.78±20.88	35.69±16.38	0.54±0.40	0.58±0.31
M825	17.39±24.12	24.26±17.17	0.93±0.41	0.33±0.27
M717	15.91±25.59	20.98± 7.78	0.90±0.66	0.89±0.47
M728	16.49±19.45	39.40±29.37	1.03±1.10	0.61±0.27
Mean	16.22±20.21	27.92±18.39	1.01±0.85	0.91±0.95
n	84	84	83	83
signif- icance ¹	ns	p<0.001	p<0.001	p<0.001

¹ analysis of variance between animals on logarithmically transformed data

noradrenaline and adrenaline 2-3 times those measured in other lambs. Restraint stress is known to be a potent stimulus to catecholamine release in the rat (Bühler *et al.* 1978). The importance of the sampling stressor in catecholamine physiology is clearly illustrated by this result. The lactic acid and cortisol levels in lamb M720 are not high in relation to their respective populations over all lambs (Table 6-5). Any sampling-generated increase in these compounds would tend to occur after blood withdrawal.

Sampling Order

When an analysis of variance was carried out with sample order within each sample set as a covariate no significant trends were revealed in any measured substance. However when the first sample collected in each sample set is compared to the remaining 5 a significantly lower ($p<0.01$) cortisol level was found in the samples collected at the beginning of each sampling (17.76 ± 8.83

ng ml⁻¹ versus 29.95±19.16 ng ml⁻¹). No differences were detected in the remaining substances. In any event it is likely that lactic acid was little effected by the sampling while it is predicted that catecholamine levels would be affected to an equal degree in individual subjects given the absence of any appreciable stimulus-secretion delay.

It is not possible to quantify the contribution of the experimental procedure itself to the release of the measured substances. Despite the precautions taken it is likely that both catecholamines and to a lesser extent cortisol may reflect some degree of sampling stimulus. With respect to the catecholamines this effect is likely to be relatively constant across the experiment and within any sample set, though individual lambs may differ in response.

Blood Splash (Ecchymosis) Score

The mob exhibited little blood splash. Five carcasses were scored as having a slight degree of splash (blood splash score 1), all others had no blood splash (score 0). The 7 cannulated lambs were all scored as 0. The probability of this eventuality given the distribution of blood splash scores in the 19 non-cannulated lambs is not significant ($p>0.2$) however using the exact probability tables.

DISCUSSION

The biphasic cortisol secretion seen in these sheep approximates, in shape if not in timing, that observed in other non-ovine species under various circumstances (§5.5). The phenomenon does not however appear to have been described outside the laboratory in a situation commonly encountered by domestic stock. It confirms the existence of 2 distinct release pathways since the high rate of secretion overnight cannot be ascribed to any new source of disturbance that was not present at the time of the postwash decline in cortisol. To the human observer the opposite was the case since general environmental noise and disturbance were much reduced. Diurnal rhythms cannot be

evoked to explain the overnight surge in cortisol levels since maximal release is not expected until the early morning-daylight hours (McNatty *et al.* 1972) and in any case diurnal rhythms are disrupted by significant environmental stimuli or changes in the surroundings (McNatty *et al.* 1972, Holley *et al.* 1975).

The genesis of the biphasic response is uncertain. It may have been associated with a single event - the water bath would be the most likely stimulus. However if a single event can cause such a discharge pattern it might have been expected that earlier events would have also activated the system preventing such a marked drop in cortisol output because of the operation of second phase release at the time. Alternatively the various stressors of the preceding 8 hours could be considered as a single continuous stimulus which on sudden termination sets in motion the second cortisol release phase. If this interpretation is correct then questions arise about the control mechanisms of cortisol release as proposed by groups such as Yates *et al.* (1971) in which the multiphase release of corticosteroids is explained by a rate sensitive followed by a proportional release control (§5.5). In the present experiment the suppression of cortisol release appears to be related to cessation of environmental stimuli, rather than to any rate of change in the level of cortisol up to this point. Slow proportional control may explain the decline in levels seen after 1.15 am. The inhibition of cortisol release after the water bath could be driven either by the sudden removal of surrounding stimuli on the secretion of corticotrophin releasing hormone, by feedback inhibition of circulating corticosteroids on release mechanisms no longer subject to stimulation or by the unleashing of inhibitory neuronal circuits in the complex corticotrophin releasing hormone control system (Gann *et al.* 1978).

It has become clear that different stressors are received, integrated and then stimulate or inhibit the release of corticotrophin by distinct but not mutually exclusive sets of nervous structures. Only the effect of haemorrhage or

hypovolaemia on corticotrophin release is understood in any detail - and then only under very carefully controlled laboratory conditions (Gann *et al.* 1978). The present data with a very complex mixture of stressors are impossible to interpret in any but a speculative manner. Nevertheless the observations are important since they represent the adreno-corticol response in a practical situation for which control theory must ultimately account.

The most obvious feature of the catecholamine profiles was their comparative lack of change over the experiment. It is almost certain that the nature of catecholamine release, uptake (§2.5) and decay in whole blood (§5.2) has prevented the detection of significant elevations of catecholamines in the plasma of the experimental lambs, for example during yarding and washing. Due to the short half-life of catecholamines in the blood the levels will have substantially or entirely returned to baseline by the time of sampling only leaving the effects of a comparatively constant background of non-specific stressors. The fluctuation in noradrenaline content in several sample sets is of interest since it was not accompanied by any change in adrenaline. While it is possible that a selective noradrenergic stimulation has occurred (Frankenhaeuser 1975) it might also be speculated that some delay in the perfusion of synaptic cleft noradrenaline of the sympathetic nervous system into the bloodstream after the plasma adrenal medullary response had been extinguished, could also cause the observed effect.

One of the interesting features of the results of the transport experiment was the high degree of consistency of the changes in the measured variables over the experiment. The overall results do not indicate any undue pressure with which the physiological mechanism of the experimental lambs could not cope and presumably this is the experience of the great majority of lambs sent to New Zealand abattoirs for slaughter.

Stock Holding Time at Abattoirs

A minimum holding time of 18-24 hours prior to killing of cattle and sheep is now mandatory at New Zealand abattoirs for a number of reasons.

1. Sufficient emptying out time is claimed to be necessary to reduce carcass contamination by gut contents during processing.
2. Time is permitted for the stock to recover from the fatigue resulting from transportation and handling.
3. Adequate time is allowed for ante-mortem veterinary inspection and the expression of any pathological conditions requiring carcass condemnation.
4. With sufficient animals held at the abattoir, killing is less likely to be held up for reasons relating to the supply of stock and reduces the need for work outside normal working hours.

There are some disadvantages.

1. The capital cost of the extra facilities to hold the stock.
2. Delays in killing results in a deterioration in the condition of stock in the holding pens and increases carcass losses.
3. The spread and increase in the level of salmonella infections.
4. Increase in the incidence of dark cutting beef in cattle carcasses.

The data collected in the present experiment enable some comment to be made on the question of recovery from the transportation. Cortisol reached its low point for the experiment at 5 pm on the day of arrival some 50 minutes after penning. It is probable for the reasons already discussed that the overnight peak is a

function of the central corticosteroid release system rather than to any reaction to the environment over that time. Lactic acid fell rapidly after the wash peak and was essentially at baseline by 7.45 pm less than 4 hours after penning. Nor-adrenaline was close to its low point by midnight while adrenaline changes were non-significant, and both catecholamine concentrations were negligible at all times in relation to the effect of electrical stunning. Thus although after midnight there is a tendency for all substances to fall further a case can be made for recovery in endocrine terms within 4-8 hours of penning. The activation of all these systems in mustering the animals up to the foot of the slaughter chain negates any further small decreases seen by holding the stock for longer periods of time. Complete return of the pituitary-adrenal axis to normal in a strange environment is not likely for weeks (§6.6) but holding stock for long periods without access to food or water will eventually cause a rise in the activity of the endocrine system. The results however do not indicate that the 24 hour holding period is stressful to these lambs. Further similar experiments will be necessary however to determine the responses of stock transported over longer distances or deprived of food and water for varying periods prior to transport.

The problem of determining an optimum stock holding time is essentially one of defining what is a "rested" animal. If the issue is one of animal welfare then earlier killing may be possible (if this was the only reason for the holding time). If ultimate meat quality and muscle biochemistry were the overriding concern then it should be possible to define the desirable characteristics and carry out a research programme to determine an optimum holding period in these terms. It is quite likely of course that different objectives may have different optimal holding times but it seems important to assess different factors scientifically in order to deal with future eventualities on a rational basis.

6.8 THE VALUE OF BLOOD HORMONE "STRESS" ASSESSMENT AS A RESEARCH TOOL

The consideration of the effect of stressors in domestic animals is relatively recent. Previously only laboratory species have been studied by behavioural psychologists but it has become increasingly apparent that some modern systems of intensive production may place strain on domestic animals particularly where associated with high stocking density. There is also reason to believe that many day-to-day management procedures including mustering, shearing, drenching and transport may inflict emotional distress on susceptible stock (Kilgour 1978a). The effect may not be important in most cases but imposed at certain critical periods in the reproductive cycle or in combination with exposure to pathogens may precipitate a significant depression in production.

Fraser *et al.* (1975) supplied the following definition of stress applicable in agricultural and veterinary sciences.

" An animal is said to be in a state of *stress* if it is required to make abnormal or extreme adjustments in its physiology or behaviour in order to cope with adverse aspects of its environment and management. A husbandry system can be said to be *stressful* if it makes abnormal or extreme demands on the animals. Finally, an individual factor such as a low temperature, may be called a *stressor* if it contributes to the stressful nature of a system of husbandry. "

While there can be debate as to what constitutes abnormal or extreme adjustments the term "stress" should certainly not be used to imply any measurable parameter which summates various reactions to any set of circumstances.

The scientific assessment of the effect of handling stressors on animals has been advocated in many quarters, particularly in view of the debate surrounding animal welfare. The objective

assessment of physiological or behavioural responses offers an alternative means to measuring production variables (which are in any case complexly related to other factors). It then becomes possible to recommend means of avoiding distress commensurate with economic and management realities. To date the results have been mixed and mostly disappointing.

Neither behavioural or physiological measures of the effects of stressors can be deemed satisfactory. Overt behavioural responses of stock, like physiological changes in the blood are altered by the presence of the investigator, his methods and approach, and continual appreciation of individual variation in behavioural responses is necessary. Behavioural observations are still to some degree handicapped by the lack of adequate knowledge of the normal behaviour patterns of stock. The same of course can be said of many physiological variables.

Physiological stressor assessment is potentially more objective and more sensitive than behavioural measures, but fraught with difficulties of its own. Some of these relate to interanimal variation (genetic diversity, previous handling experience) or to the timing of sampling. A knowledge of diurnal and seasonal fluctuations is essential to avoid errors associated directly with the normal dynamics of the intact system (Squibb 1974). Many physiological changes are transient because the mammalian body is kept in a steady state by an array of negative feedbacks. This means that the timing of the sample is critical to the final interpretation of any cause-and-effect phenomenon, as has been illustrated in the transport experiment. Generally a number of blood samples must be collected to assemble an accurate conception of the hormonal responses since 2 samples collected in an interval of time may give quite opposite impressions. The artefacts caused by sampling stressors have also been demonstrated in the present experiment. A further problem is that emotional responses resulting in systemic and endocrine perturbations are not necessarily related to specific psychological stimuli but may be caused by a wide range of novel stimuli presented suddenly and

simultaneously to the animal. Blood substances have physiological functions to serve, even though they may initially be released in response to the stimulus the experimenter is anxious to study. The problem is to minimize the physiological considerations so that valid data with psychological implications can be obtained.

In addition to the foregoing it is clear that the short and long term controls on the secretion of various substances are complex and different stressors cause different response patterns. While the Selye General Adaption Syndrome is useful in predicting many physiological responses, the idea of a basic unitary reaction in response to a large number of disparate stimuli is an over simplification. Catecholamines and corticosteroids, the immediate and the delayed responses of Cannon and Selye, are increasingly being used as a measure of emotional arousal in behaviour studies of man, laboratory animals and domestic stock (Reid & Mills 1962, Frankenhaeuser 1975, Henry *et al.* 1975a).

Behavioural responses do not always permit quantitative or even qualitative distinction between sequential events. In the transport experiment (§6.7) behavioural changes were not studied in detail but alterations in the blood levels of the measured substances could not always be predicted from the lamb behaviour. On the other hand the second phase elevation in cortisol levels may constitute a preprogrammed response set in motion by a previous experience of the lambs which had long passed from the animals' consciousness. The measurements of blood variables even considering the additional imposition of experimental procedures enables a continuous and complex process like animal transport to be dissected and problem areas identified. Physiological data are important too in another respect; they provide links between an event and the consequences of that event on ultimate production.

In summary, 3 main problem areas exist in the application of laboratory theory to the solution of practical problems of agricultural origin.

(a) Selection of the Blood Component(s) to Measure

Any measure of bodily function has many variables and a discussion of all influences, even if they were known, is impossible. Emphasis must therefore be placed on those variables and influences with management application. Sometimes the hormones or blood substances determined have little or no relevance to the problem under study. This situation most commonly arises where an attempt is made to measure a large number of substances simultaneously (eg. the full range of blood minerals in a study of transport hypocalcaemia). While this approach may be valid in an initial screening trial careful selection of the measured parameters should be made with the reason for the study the determining factor. For example lactic acid is a measure, primarily of an animal's response to exercise and anaerobic metabolism (though also to catecholamines and nervous arousal). Since preslaughter lactic acid levels have a role in the postmortem history of the carcass (§6.3) lactic acid is a good indicator of pre-slaughter processes from a meat quality point of view.

Some physiological functions of economic importance, such as lactation or nutritional intake are not commonly associated with any specific blood substances affected under conditions of stress. However, knowledge of the physiology of the subject will usually suggest an appropriate parameter(s). A large number of hormones, minerals and other blood components are perturbed under circumstances leading to behavioural and physiological adaptations.

(b) Sampling Conditions

Difficulties arising in sampling occur in several categories. First, the timing and the number of samples taken over an experiment are critical. If the time span involved is long then many samples may need to be taken to obtain an idea of the processes occurring. Too often only 2 samples are taken; one at the start, and the other at the end of the experiment. Usually this is quite inadequate. The choice of a sampling regime must be based on a sound understanding of the dynamics of the substance under study. The second area of difficulty is an example of the Heisenberg Uncertainty Principle. That is, the process of obtaining a blood sample will in many cases perturb the variable being measured. It is often possible to avoid or minimize the effect if prior consideration is given to the problem.

(c) Basic Physiologic and Biochemical Background

The physiology and biochemistry of the substance is usually not understood (or at least not discussed) in sufficient detail to obtain useful results in terms of the achievement of a practical goal. Most substances have complex homeostatic controlling mechanisms. The levels in the blood at a particular instant in time may be either high or low depending on the nature of the stressor and its duration, the stores of the material within the body, its release and uptake. A thorough physiological understanding of the problem in terms of the substance chosen, the form of the experiment and the interpretation of the results is the most important prerequisite for any systemic study of "stress".

PHYSIOLOGICAL AND ENDOCRINE EFFECTS OF THE ELECTRICAL STUNNING AND SLAUGHTER OF LAMBS

CHAPTER 7

7.1 INTRODUCTION

Traditionally, fully conscious sheep in New Zealand have been slaughtered by a transverse incision ("sticking") of the extended neck simultaneously severing the trachea, oesophagus, jugular veins, carotid arteries and the spinal cord at the occipito-atlantal junction. The recent requirement of the Ministry of Agriculture and Fisheries Meat Division, based on European Economic Community directives, that all sheep and lambs in New Zealand should be stunned before slaughter has meant a re-adjustment in the approach to and the techniques of slaughter in order that the animals are presented as best to simplify the stunning operation, that stunning to the point of unconsciousness be rapid and that blood letting be efficient. The physiological responses of lambs to electric stunning and slaughter are also of wider significance regarding humane handling (Kilgour 1978b) and ultimate meat quality (§6.6). But apart from these considerations there is associated with electrical stunning the development of haemorrhages in varying degrees in lamb carcasses which is not seen in animals slaughtered by sticking alone (Warrington 1974). Strictly speaking the term ecchymosis is applied to the large diffuse haemorrhages and petechiae to the small (1-10 mm) round or oval spots. For the purposes of this discussion the commonly used term "blood splash" will be used to cover all types of

carcass haemorrhage. Blood splash shows an anatomical variation in its incidence being more common in the gall bladder, heart, lung, diaphragm, abdominal wall, dorsal muscle and loin and results from the rupture of blood vessels at or within a few seconds of electrical stunning (Tweed *et al.* 1931). Usually only a small number of animals are affected but the lesions are unsightly in severe cases. High rejection rates of cattle carcasses on account of blood splash have been reported from Australia (Charles 1960, Whittem *et al.* 1960) but data on the commercial significance of the problem on an international scale are not readily accessible.

The massive release of catecholamines in response to electrical stunning found in the transport experiment (§6.7) is examined in detail in the present chapter. Electron micrographs of adrenal medullae from both stunned and non-stunned animals are first examined (§7.2) followed by a description of the plasma profiles of cortisol, lactic acid and the catecholamines in lambs subjected to stunning (§7.3). Experience with the electrical stunning of lambs at the Auckland Farmers' Freezing Company Horotiu abattoir and elsewhere has revealed marked differences in the level of blood splash between mobs (Frazerhurst 1976). Among the explanations of these differences are the suggestions, generally unsubstantiated, that the degree of prior "stress" is a causative factor, an increasing level of psychological arousal being associated with an increasing level of blood splash (Tweed *et al.* 1931, Charles 1960). Seven mobs of lambs were studied for behavioural and physiological responses to the abattoir environment (§7.4) and the particular stressors bearing on these mobs examined for any linkage between their handling history and the incidence of blood splash (§7.5). Finally the effects of adrenergic blocking drugs in suppressing blood splash were investigated and related to the physiological basis of the lesion as it is presently understood (§7.6).

7.2 THE EFFECTS OF ELECTRICAL STUNNING ON THE CATECHOLAMINE CONTENT AND THE MICROSCOPIC STRUCTURE OF THE OVINE ADRENAL MEDULLA

INTRODUCTION

Adrenal medullary catecholamine depletion is difficult to achieve experimentally without the use of pharmacological agents such as reserpine. While newly formed noradrenaline is preferentially secreted from both the adrenal medulla and the sympathetic nerves (Stjärne 1971) it is supplemented by the recapture and reuse of released catecholamines (§2.4). Furthermore large quantities of catecholamines are held within the storage vesicles of the sympathetic neurones and chromaffin cells (§1.4). This large catecholamine storage capacity together with the reuptake and rapid biosynthesis of catecholamines is reflected in the sustained adrenal output of both noradrenaline and adrenaline that occurs even 3 hours after acute haemorrhage ($\frac{1}{3}$ blood volume depletion) in dogs (Zileli *et al.* 1974) while repeated electroshock therapy in humans (2 or 3 times weekly) does not cause any diminution in the plasma adrenaline response (Havens *et al.* 1959). A few studies have however detected catecholamine depletion after chronic sympathetic stimulation. In rats 5 hours of continuous vigorous exercise reduces the adrenal adrenaline content with a much greater reduction occurring when tyrosine hydroxylase is pharmacologically blocked (Gordon *et al.* 1966). Long term exposure to climatic heat has been found to cause a marked fall in human urinary catecholamines and the cause has been attributed to an exhaustion of adrenal stores (Sulman *et al.* 1977).

The rapid release of large quantities of catecholamines into the bloodstream engendered by electrical stunning raises the possibility of a significant depletion of adrenal catecholamine contents. An experiment was therefore undertaken to examine the biochemical and morphological consequences of electrical stunning on the adrenal medulla.

METHODS

Experiment 1 Adrenal glands were collected from thirty 6-8 month old Romney lambs after slaughter at an abattoir. Both adrenal glands were collected where possible but occasionally one was lost or damaged during the carcass evisceration. Half of the group was stunned prior to slaughter with an electrical Stunner (Stampco Engineering Pty Ltd, Carlton South, Victoria, Australia). The current was applied to the occipital region of the head through 2 pointed parallel electrodes 50 mm apart (100 V, 0.75 amp, 50 Hz for 1-2 seconds). The adrenal glands were collected within 5 minutes of slaughter and preserved on ice. Within an hour any adhering connective tissue was removed and one gland from each lamb was weighed, homogenized in 5 ml of 0.4 N ice-cold perchloric acid and centrifuged at 15 000 g for 30 minutes. 0.5 ml of the supernatant was extracted for the catecholamine content, (§4.3) the eluate diluted 10 fold and analyzed fluorimetrically for dopamine, noradrenaline and adrenaline (§4.4). A small medullary tissue sample (approximately 0.1 g) was dissected from the centre of the second adrenal gland from each lamb, weighed, homogenized, extracted and analyzed as described.

Experiment 2 Adrenal glands were collected from 8 month old Romney lambs within 5 minutes of slaughter at an abattoir. Eleven of these lambs were electrically stunned and 8 were non-stunned. A thin slice of medullary tissue was immediately dissected from the centre of each gland and fixed in glutaraldehyde. Ultrathin sections for electron microscopy were prepared from these tissue samples using the methods of Hayat & Giaquinta (1970) by Mr N.G. Leet of the Meat Industry Research Institute of New Zealand.

RESULTS

Experiment 1 The mean weight of the whole glands analyzed was 1.34 ± 0.27 g ($n=25$). The catecholamine content was predominantly adrenaline with lesser amounts of dopamine and noradrenaline (Table 7-1).

Table 7-1
Catecholamine Contents of Adrenal Glands Collected from
Stunned and Non-stunned Lambs

		Dopamine $\mu\text{g g}^{-1}$	Noradrenaline $\mu\text{g g}^{-1}$	Adrenaline $\mu\text{g g}^{-1}$
Whole Glands				
stunned	(13)	56.4 ± 44.6	58.5 ± 23.8	297.4 ± 70.9
non-stunned	(12)	50.3 ± 19.6	40.2 ± 14.1	380.5 ± 114.2
Medullary Slices				
stunned	(12)	351.8 ± 202.7	573.2 ± 274.5	2568.0 ± 612.7
non-stunned	(15)	323.6 ± 146.5	469.0 ± 186.5	2891.7 ± 728.1

numbers analyzed in each group given in parenthesis

The concentrations of these catecholamines in both the whole glands and in the medullary tissue are similar to those reported previously in the sheep (Shepherd & West 1953, West 1955) although in the whole glands the mean ratio of adrenaline to total adrenaline plus noradrenaline is higher (0.85 ± 0.04 compared with 0.67 (Shepherd & West 1953)). Electrical stunning had no significant effect on the whole gland or medullary tissue concentrations of any of the 3 catecholamines.

Experiment 2 The major ultrastructural features of the ovine adrenal medulla are similar in many respects to those described in the dog (Carmichael & Blair 1973), hamster (Grynspan-Winograd 1975), pig (Palmer 1978) and other species. The chromaffin tissue shows the typical features of a large storage vesicle population

with the cells separated by numerous blood vessels, connective tissue and both myelinated and non-myelinated nerve fibres (Plate 7-1a,b). Distinct noradrenaline- and adrenaline-containing cells that may be morphologically distinguished after glutaraldehyde fixation have been identified in other species notably the hamster (Grynszpan-Winograd 1975) and dissimilar cell populations were also observed in the present study. Noradrenaline cells contain intensely electron-dense granules often displaced from the centre of the surrounding vesicle (Plate 7-2a) while the adrenaline-containing vesicles are characterized by a light to moderate electron density and are surrounded by a translucent halo (Plate 7-2b). The observation that the great majority of the chromaffin cells examined contained adrenaline supports the biochemical data.

No clear differences were observed between medullary tissue from the non-stunned (Plate 7-1a) and the stunned (Plate 7-1b) lambs. However chromaffin cells from the stunned lambs appeared to be more vacuolated and there was a much larger quantity of fibrous material in the intercellular and vascular spaces. These were however differences of degree only and it is apparent that a much more extensive survey would be needed to confirm initial impressions. The simultaneous expulsion of a large quantity of catecholamines into the bloodstream after electrical stunning might constitute an interesting means to study the still unresolved mechanism of catecholamine release (§2.5). In the present study no evidence comparable to that seen in the hamster adrenal medulla (Grynszpan-Winograd 1975) was found to support the exocytosis theory of catecholamine release (§2.5). In general however the electron microscopy confirmed the biochemical results that large as the stunning release might be the catecholamine stores in the adrenal are considerable and are not significantly affected.

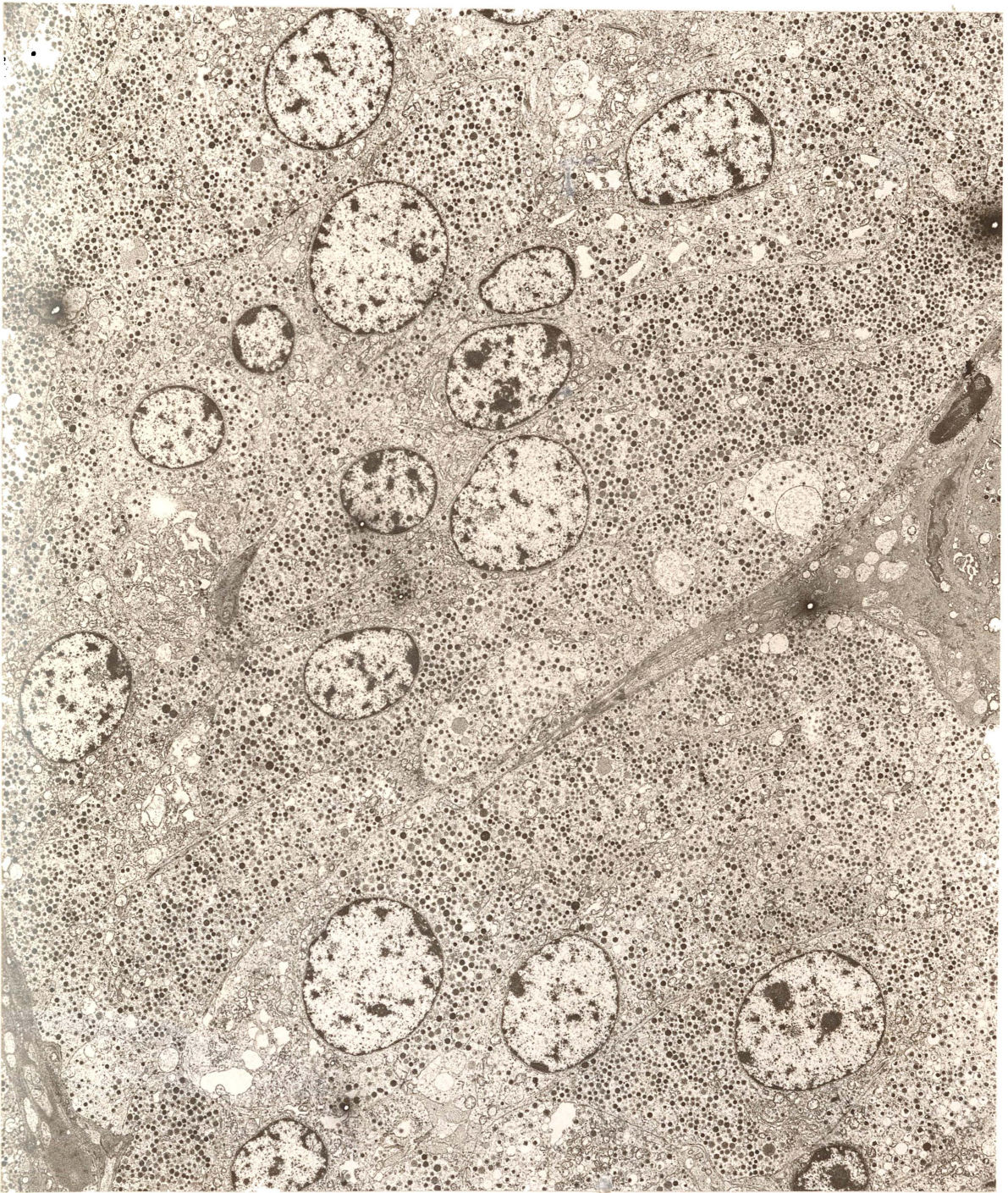


Plate 7-1a. Lower power electron micrograph of adrenal medullary tissue from a non-stunned lamb (x 2 000)

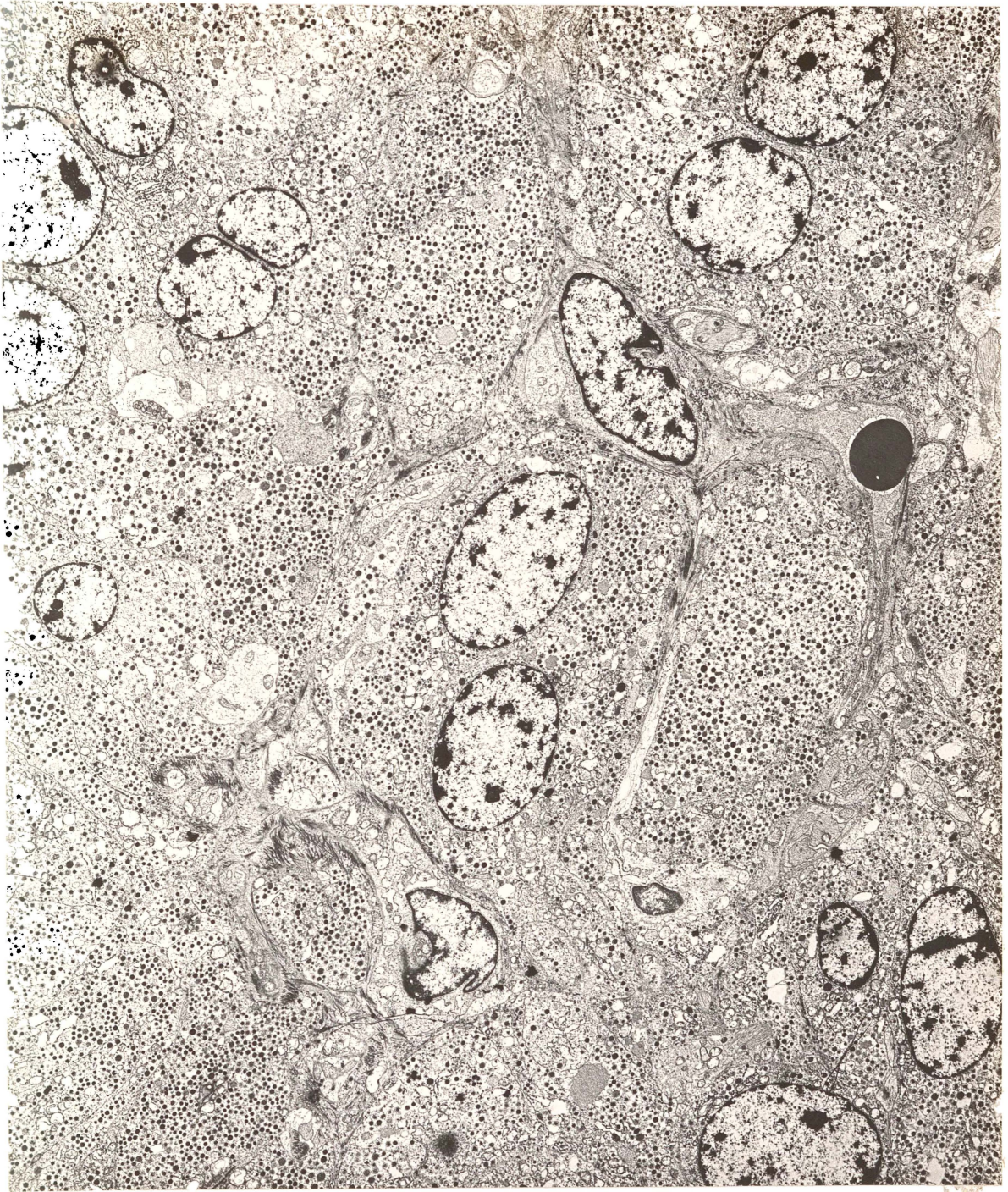


Plate 7-1b. Lower power electron micrograph of adrenal medullary tissue from a stunned lamb (x 2 000)

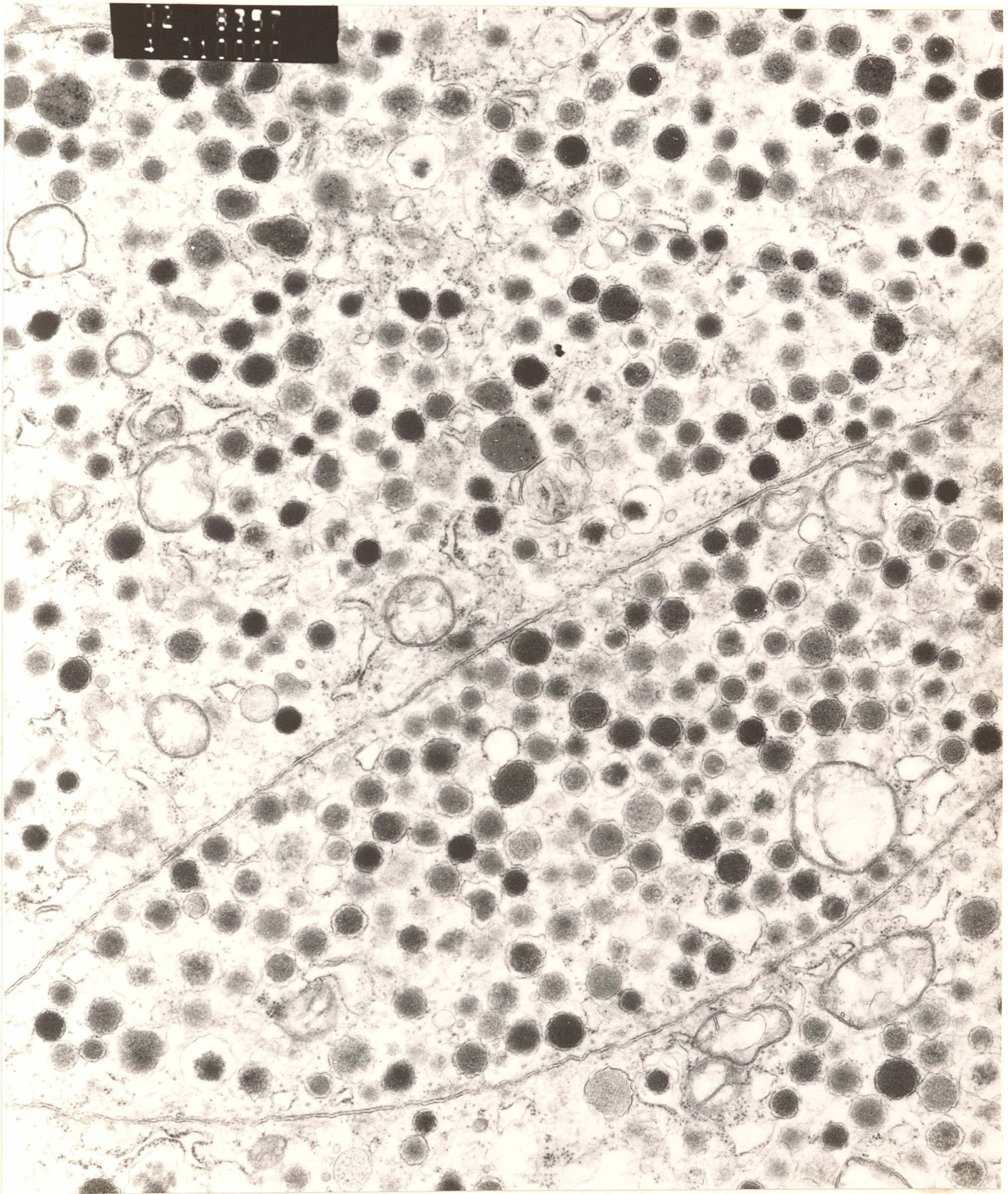


Plate 7-2a. Noradrenaline-containing area of the adrenal medulla from a non-stunned lamb (x 10 000)

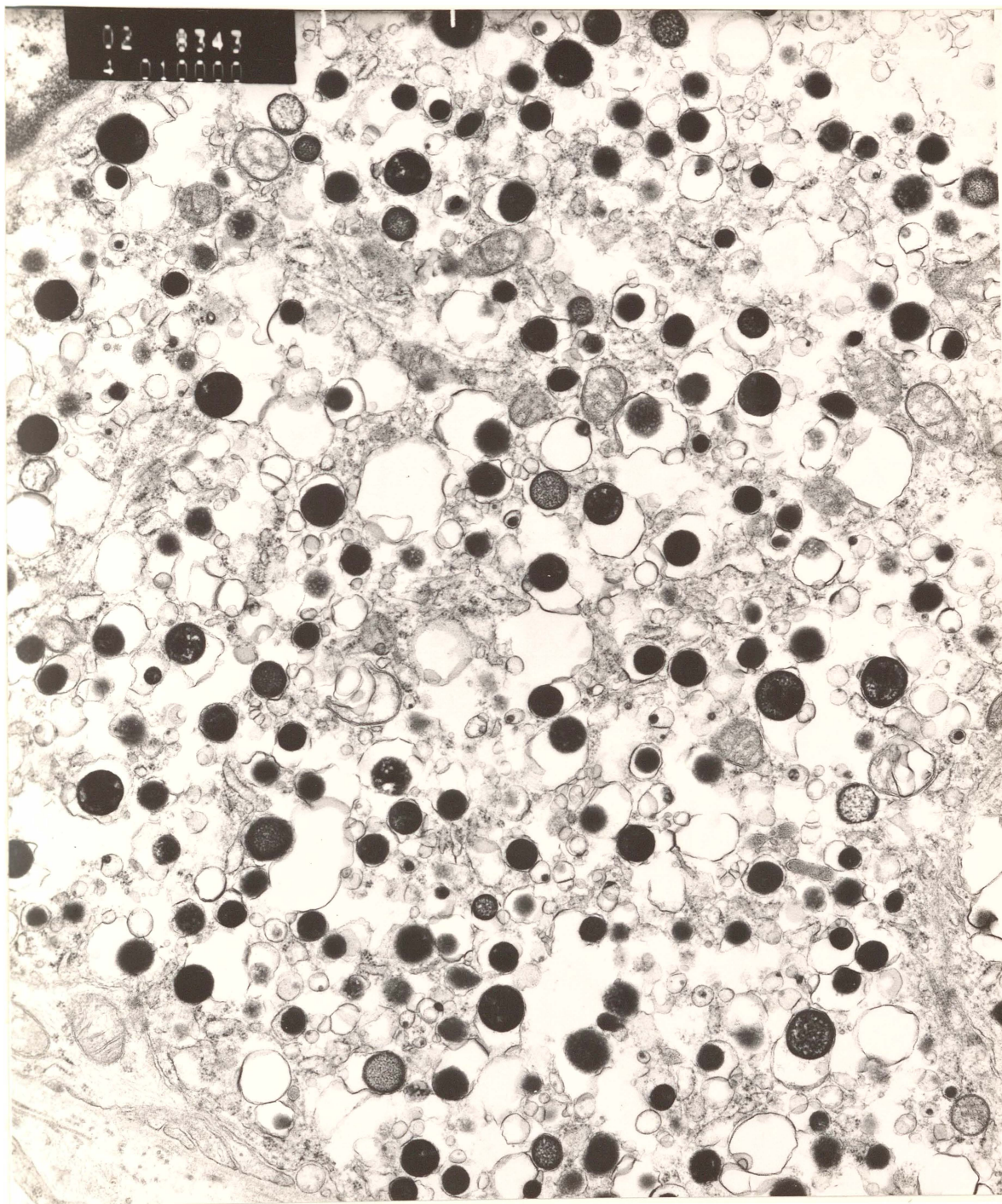


Plate 7-2b. Adrenaline-containing area of the adrenal medulla from a non-stunned lamb (x 10 000)

7.3 METABOLIC AND ENDOCRINE CONSEQUENCES OF ELECTRICAL STUNNING

INTRODUCTION

While some knowledge has been accumulated on the hormonal and metabolic consequences of electrical stunning of slaughter stock little information is available on the effects in animals stunned but not slaughtered. Medullary noradrenaline or adrenaline output may be selectively increased or decreased by low level electrical stimulation of specific sites in the central nervous system (Carmichael *et al.* 1975). These data are not applicable however to the generalized cerebral shock caused by electrical stunning where simultaneous stimulation of all control structures is likely. The most relevant body of data is that arising from the experimental investigation of electroconvulsive therapy of humans undergoing psychiatric treatment. Although electroconvulsive therapy has long been used in the treatment of depression the physiological basis of its efficacy is poorly understood. Nevertheless some short term metabolic and endocrine effects have been observed attributable to the acute functional disturbances attending the shock itself.

Significant elevation of both plasma noradrenaline and adrenaline follow unmodified electroconvulsive therapy and may become greater with successive treatments (Weil-Malherbe 1955, Havens *et al.* 1959). Lesser responses are seen in modified treatment where anaesthetics and/or neuromuscular blocking drugs are employed. Associated with these endocrine changes are a marked increase in blood pressure and changes in the heart rate (Gravenstein *et al.* 1965). The increase in blood pressure is significantly correlated to the increase in the circulating noradrenaline levels (Gravenstein *et al.* 1965) while in the dog the pressor response has been attributed solely to the increased levels of circulating catecholamines since spinal anaesthesia (which blocks the catecholamine release) or α receptor blockade abolish the pressor effect (Anton *et al.* 1977). Other hormonal responses to electroconvulsive therapy are reported to include

increased growth hormone (Schalch 1967) and, in some male patients, increased luteinizing hormone and follicle-stimulating hormone (Ryan *et al.* 1970). Ylikorkala *et al.* (1976) found no consistent changes in the concentrations of follicle-stimulating hormone, luteinizing hormone, thyroid stimulating hormone, growth hormone or cyclic adenosine 3',5'-monophosphate although individual increases in these substances were noted. Consistent increases in corticotrophin and cortisol production were evident in all subjects although these authors suggested that non-specific stressors associated with the procedure were responsible rather than an electrically mediated release of corticotrophin releasing hormone. In the rat increasing the intensity and the duration of the electric shock has the effect of increasing the corticosterone response (Friedman *et al.* 1967).

Marked brain metabolic changes and an increase in energy expenditure accompany electroshock in the rat. Decreased brain glycogen, glucose, phosphocreatine, and adenosine triphosphate and increased concentrations of lactic acid, adenosine diphosphate and inorganic phosphate occur (Klein & Olsen 1947). Brain lactic acid increases to a maximum at 2 minutes after electroshock in the rat falling to a depressed level at 30 minutes and returning to the resting value at 60 minutes (Leonard 1971). Of the β -adren-ergic blockers studied propranolol was the most effective in reducing both resting lactic acid levels and the extent of the increase in concentration after electroshock. The mode of action of propranolol could not be ascertained from the study but could be due either to an increase in glucogenesis or glycogenesis within the brain or to a decreased rate of glycolysis leading to a reduction in lactate formation from glucose. It may be a significant factor in the metabolic changes caused by electroshock that cyclic adenosine 3',5'-monophosphate urinary excretion is increased in patients undergoing electroconvulsive therapy and is at least partly independent of any change in catecholamine activity (Hamadah *et al.* 1972). A higher but statistically non-significant increase in brain cyclic adenosine 3',5'-monophosphate was found in

pigs electrically stunned before bleeding compared with those slaughtered by bleeding alone (Ono *et al.* 1976).

The present experiment was designed to examine the changes in lactic acid, cortisol and the catecholamines brought about by electric stunning of intact lambs.

METHODS

Carotid cannulae were implanted in two 6-7½ month old Dorset Horn lambs (M730 and M795) by the method described in §5.2, 48 hours before the experiment. On the day before slaughter the lambs were transported 1 km to the abattoir and left overnight in the holding pens. A blood sample was collected from each lamb via the carotid cannula 1 minute before stunning and then at the following times after stunning: 20 second, 1, 2, 5, 10, 30, 60 and 120 minutes. The sampling time was generally less than 20 seconds. A second stunning was then carried out and the sampling procedure repeated.

RESULTS

After stunning the lambs were unconscious for up to a minute. With some difficulty they generally stood up, generally within 3 minutes of stunning, after which no overt behavioural effects of the treatment were seen. Since the assay results from the 2 lambs were very similar both qualitatively and quantitatively the results have been pooled for graphing (Figure 7-1). Lactic acid increased very rapidly after stunning to a maximum between 2-10 minutes followed by a decline back to control levels over the subsequent 2 hours. A very similar response was observed on the second stunning though in both cases the lactic acid level rose further than previously. Cortisol, after a small rise at stunning, did not change significantly until 5 minutes post-stunning but then commenced to rise to a maximum at 30 minutes. Levels remained high until the second stunning when, after a small further fluctuation, a slow decline occurred. Levels were still above the control concentration range at the termination of blood

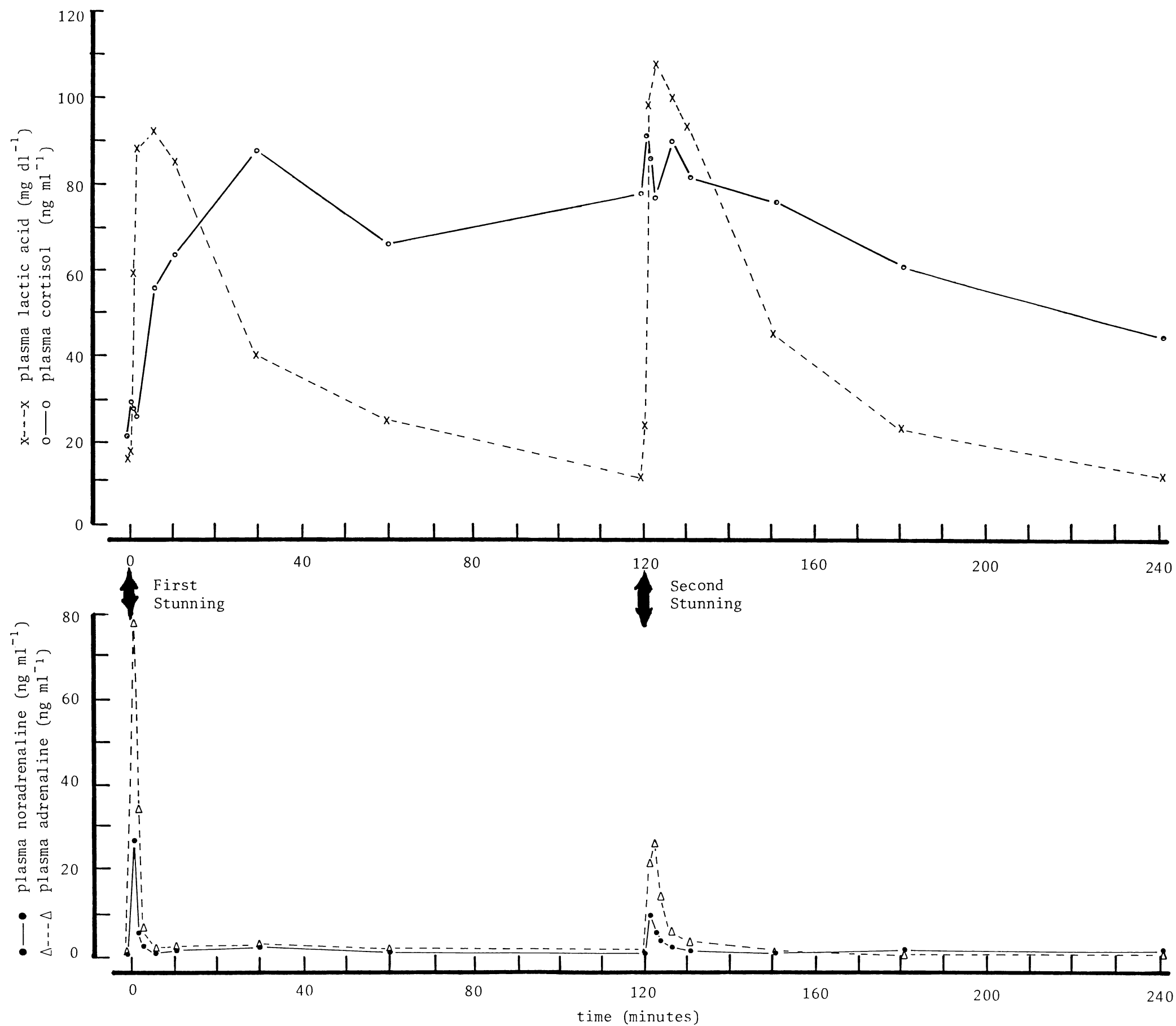


Figure 7-1. Mean plasma lactic acid, cortisol and catecholamine profiles of 2 lambs subjected to electrical stunning at 0 and 120 minutes.

sampling. The catecholamines showed a very sharp increase to reach maximum levels within 20-40 seconds of stunning, followed by an almost equally rapid decline to close to prestunning values. The same qualitative changes occurred at the second stunning but the maximum adrenaline levels were reached slightly later at 1 minute post-stunning. Both noradrenaline and adrenaline peak concentrations were less than half those following the initial stunning.

DISCUSSION

When the physiological effects of electrical stunning are considered it is necessary to distinguish between primary and secondary effects. That is, between those directly connected with the electroshock and those dependent on the nervous discharge such as muscle contraction, cardiovascular changes and psychological distress.

It is clear that electrical stunning causes a very large increase in lactic acid within the tissues of stunned lambs since its effusion into the blood is substantial and closely related to the time of stunning. The cause of this phenomenon is probably attributable to the metabolic demands (including glycolysis) of the intense muscular contraction characteristic of electrical stunning (§7.5). However the large and immediate adrenaline release must also augment and extend the life of the elevated blood lactic acid levels (§5.5). The poststunning lactic acid profile may also be influenced by a degree of hypoxia since electroconvulsive therapy is said to need careful supervision in relation to apnea and may include the use of supplementary oxygen (Havens *et al.* 1959, Hamadah *et al.* 1972).

The characteristic stimulation-secretion delay (§5.5) of cortisol is well illustrated by the results and contrasts with the almost immediate release of the catecholamines. This delay also enables an interesting observation to be made in relation to the effects of electroshock on the partitioning of body fluids. Immediately after electroshock therapy in man there occurs a short-lived

haemoconcentration of the order of 5-10% (Reichlin & O'Neal 1962). In the longer term fluid retention by the body occurs and results in extracellular fluid expansion and a gain in body weight (Brown *et al.* 1963). The small peaks in cortisol on both stunning occasions probably reflect such a change rather than any immediate change in secretion rate. It is not possible to distinguish between the direct effects of the electrical stunning on corticotrophin release and the indirect effects of the psychological reaction of the lambs to stunning. The latter is bound to be a factor and it must also be remembered that indiscriminate depolarization of inhibitory as well as stimulatory control pathways (§5.5) in the central nervous system would occur with electrical stunning.

On the second stunning when cortisol levels were already elevated a suppression of further release is apparent. This inhibitory feedback component of the cortisol release mechanism (§5.6) was invoked by Redgate *et al.* (1974) in explanation of the failure of electrical stimulation of the infundibular area of the cat hypothalamus to cause a release of corticotrophin when circulating cortisol levels were already high. Contrary results to these were found by McHugh & Smith (1967) who showed that pretreatment with cortisol did not suppress corticotrophin release in the monkey when other hypothalamic sites were stimulated. Redgate *et al.* (1974) reconciled this discrepancy by postulating a sympathetically mediated angiotension response activating corticotrophin release via a mechanism not susceptible to steroid feedback (Gann & Cryer 1973). Renin release is increased by electroshock (Leenen & Shapiro 1974) as well as by circulating catecholamines (Davis & Freeman 1976, Henry *et al.* 1977). Redgate *et al.* (1974) proposed on anatomical and technical grounds that a sympathetic activation occurred in the experiment of McHugh & Smith (1967) which had not occurred in their own. While it is not possible to directly compare the crude electroshock trauma of slaughter stock to the precise electrical stimulation techniques used in these laboratory studies it is manifestly evident from the present study that sympathetic activation

operated in addition to corticotrophin suppression. Thus peripheral sympathetic stimulation does not automatically lead to corticotrophin release in the presence of high circulating cortisol and other explanations have to be considered. One possible mechanism might act through the inhibitory noradrenergic pathways in the central nervous system (§5.5). Dopamine (Engel *et al.* 1968, Billiet *et al.* 1970) and noradrenaline (Kety *et al.* 1967) turnover and utilization are accelerated in the central nervous system of rats and rabbits by electroshock. There is some evidence also to suggest that repeated electroshock treatment of rats lowers the neural affinity for noradrenaline uptake and may also reduce the number of uptake sites as well, thereby potentiating the potency of released noradrenaline (Hendley & Welch 1975). Some investigators propose that the modification of catecholamine release and biosynthesis is the basis for the efficacy of electroconvulsive therapy in depressive illnesses (Kety *et al.* 1967) though the interpretation of these data is still controversial (Papeschi *et al.* 1974).

The predominance of adrenaline in the catecholamine response to electrical stunning is in accordance with the proportions found in the ovine adrenal medulla (Comline & Silver 1961), and indicates that the bulk of the plasma catecholamines measured is adrenal in origin. The reduced response of the catecholamines to the second stunning was evident in both lambs. From the biochemical and micrographic evidence already discussed (§7.2) it is clear that depletion of the catecholamine content of the glands does not occur, although it could still be argued that a depletion occurs in a compartment of catecholamines reserved for immediate release. On the other hand extremely rapid re-synthesis of catecholamines in the dog adrenal medulla over 3 hours in response to a release caused by insulin-induced hypoglycaemia has been demonstrated (Zileli & Gedik 1975). In the present experiment the reduced medullary response to a second stimulus is possibly the result of physical damage to the gland itself or to higher spinal or central nervous structures.

The difference between the corticosteroids whose release is only partly neural in origin and the catecholamines can be seen in the magnitude of the difference between the stunning responses and the concentrations between stunning. While catecholamine levels are high by usual standards before and between stunnings (in the range 1-5 ng ml⁻¹ of adrenaline) the response to electroshock is quite outside the normal physiological range. The magnitude of the peak catecholamine response is much greater than that observed in humans (Gravenstein *et al.* 1965) or in dogs (Anton *et al.* 1977) after electroshock. The differences may be partly attributable to the early and frequent sampling in the present experiment compared to these other studies as well as to differences in the current strength and duration (Anton *et al.* 1977).

7.4 HORMONAL RESPONSES OF LAMBS TO ELECTRICAL STUNNING AND SLAUGHTER

INTRODUCTION

The behavioural and endocrine reactions of lambs to stunning and slaughter under commercial conditions were examined in detail at a large regional abattoir (Horotiu) and a small local research abattoir (Ruakura) over a period of several months. The level of blood splash was recorded in the carcasses of all stunned lambs and the slaughter concentrations of the catecholamines and cortisol examined for any relationship to blood splash. The data collected in this experiment were also used to investigate the suggested link between ante mortem handling and blood splash incidence (§7.5).

METHODS

Experiment 1 Seven mobs totalling 1028 lambs (27 to 588 lambs per mob) killed over a period of 3 hours from 8 am on one chain at the large abattoir were studied. All lambs were electrically stunned before slaughter and all had been trucked from the farm of origin the previous day, washed and held overnight. Details

of farm locations, distance travelled and time on the road were gathered from the truck drivers at the time of arrival. Further information was gathered later by personal contact with the farmers of origin. Behavioural observations were commenced on arrival of the lambs at the abattoir and continued until slaughter the following day. After reviewing the possible factors which might act as stressors to lambs during the preslaughter period a 2-fold approach was made to the collection of data about each of the 7 mobs.

(A) Information associated with the period before arrival at Horotiu relating to the farm and transport experiences of the animals.

- (i) farm location and topography
- (ii) size of the mob
- (iii) distance and time spent travelling
- (iv) whether woolly or shorn
- (v) breed
- (vi) size of farm paddocks
- (vii) other factors including the sequence of events on the day of mustering, whether farm bikes or horses were in general use and the use of dogs

(B) Factors relating to incidents which occurred at the abattoir - events which were classified and for which the lambs involved were marked on the head with an aerosol dye and the ear tag number noted for later identification.

- (i) on arrival at the abattoir found to have been trampled in the truck
- (ii) water inhalation during washing
- (iii) mauling by dogs

- (iv) manhandling (shifting of individual sheep by physical force)
- (v) lambs left in the stunning conveyor during stoppages for 5 minutes or longer

Blood samples of 20 ml were collected from the severed carotid arteries of approximately every third lamb on the chain, as soon as possible after gash-cut sticking and within 10-25 seconds of stunning (Thornton Mark I electrical stunner, Thornton Engineering, Auckland, New Zealand). Sampled lambs were tagged with sequential numbers and all carcasses were graded for blood splash using the Meat Industry Research Institute of New Zealand rating system (0 for no splash, 1 for the presence of one or a very few small haemorrhages and ranging up to 4 for very severe splash). Overall mob splash incidence was derived by adding all individual scores and dividing by the number of assessed carcasses. Immediately following collection in bottles containing 125 IU of heparin the blood samples were split into 2 for catecholamine (§4.4) and cortisol (§4.8) analyses. In all 395 blood samples were collected of which 188 were analyzed as being representative of the mobs and adequately covering the full range of splash scores. A further 49 samples from stunned lambs were collected a few days later at the small abattoir including samples from a group of 8 lambs which were deliberately aroused by loud noises and dogs prior to slaughter and another group of 8 lambs which were handled as quietly as possible. In addition on separate days 20 and 21 samples were collected at the large and the small abattoirs respectively from non-stunned lambs (otherwise subjected to similar handling to the stunned lambs).

Table 7-2
Summary of the Blood Collections

Collection Date	Location	Lamb Treatment	Number of Samples Analyzed
29.1.76	large abattoir	stunned	188
3.2.76	small abattoir	stunned	49
25.2.76	large abattoir	non-stunned	20
9.3.76	small abattoir	non-stunned	21

Experiment 2 To determine the time taken for medullary catecholamines to reach the carotid artery after electrical stunning and gash cutting blood samples were collected from 12 and 36 lambs on 2 separate occasions at various times between 5 and 60 seconds following stunning. A single sample only was taken from 30 of the animals, from the remainder a series of 3 sequential samples was obtained. Sample collection time was of the order of 10 seconds but varied depending on the rate of blood flow.

Statistical Analysis An analysis of covariance was carried out on the logarithmically transformed analytical data. Blood splash score was the covariate while mob and slaughter location were the main effects. Because of the unbalanced nested design of the analysis of variance the method of Cummings & Gaylor (1974) was used to test the significance of differences in the results between the 2 slaughter locations.

RESULTS

Experiment 1 The mean levels of cortisol, noradrenaline and adrenaline in the plasma collected from lambs at the 2 locations are set out in Table 7-3.

Table 7-3

Hormone Levels in Lambs Slaughtered at the Large and Small
Abattoirs (Mean $\pm$ SD)

	Large Abattoir	Small Abattoir	Significance of Difference
<u>Non-stunned</u>			
Noradrenaline (ng ml ⁻¹)	3.2 $\pm$ 3.1 (20)	3.6 $\pm$ 5.9 (21)	ns
Adrenaline (ng ml ⁻¹)	8.7 $\pm$ 9.2 (20)	4.5 $\pm$ 8.1 (21)	p=0.05*
Cortisol (ng ml ⁻¹)	40.4 $\pm$ 12.4 (13)	35.0 $\pm$ 8.9 (9)	ns
<u>Stunned</u>			
Noradrenaline (ng ml ⁻¹)	73.8 $\pm$ 40.0 (188)	40.5 $\pm$ 18.6 (46)	p<0.05*
Adrenaline (ng ml ⁻¹)	96.9 $\pm$ 51.0 (188)	60.6 $\pm$ 31.1 (46)	p<0.10 ⁺
Cortisol (ng ml ⁻¹)	63.7 $\pm$ 26.3 (114)	41.1 $\pm$ 25.4 (49)	p<0.10 ⁺

numbers analyzed in parenthesis

ns non-significant

For obvious reasons the slaughtering process is stressful and is associated with considerable adrenergic drive initiated by the trauma *per se* and by the concomitant haemorrhagic shock. This is reflected in the high catecholamine levels in the unstunned lambs at both locations. While there is no difference between locations in the noradrenaline levels there is a difference at the 5% level in adrenaline - with higher levels at the large abattoir. In the unstunned lambs cortisol did not significantly differ between locations.

But while catecholamine levels in unstunned lambs were high electrical stunning is clearly responsible for a massive discharge of medullary catecholamines. Levels of noradrenaline ranged up to 219.2 ng ml⁻¹ while the highest adrenaline concentration was

285.7 ng ml⁻¹. All measured substances were higher on average at the large abattoir than at the small abattoir but only reached significance at the 5% level in the case of noradrenaline. The effects of the deliberate agitation of a group of 8 lambs prior to stunning and killing at the small abattoir were reflected in cortisol levels of 79.5±22.5 ng ml⁻¹. On the other hand lambs treated as quietly as possible produced lower slaughter cortisol concentrations (13.3±3.0 ng ml⁻¹, n=8) than seen in lambs handled under the usual conditions.

While the variation between mobs was considerable and statistically significant there was no relationship between cortisol, noradrenaline and adrenaline levels on the one hand and carcass blood splash scores on the other (Table 7-4). The exceptionally high adrenaline levels of mob 4 are of interest. This mob also had the lowest incidence of blood splash. When the catecholamine concentrations (mob 6 only) are divided into the corresponding carcass splash score groups (Table 7-5) again there are no significant trends discernible. In this case the most haemorrhaged carcasses were associated with the highest mean adrenaline concentrations.

Experiment 2 The discharge of catecholamines from the adrenal medulla in response to electrical stunning and the transit time to the carotid artery amounts to a delay of 10-15 seconds before appreciably higher catecholamine levels may be found in the carotid plasma (Figure 7-2). After this levels remain high for as long as the blood flow continues.

DISCUSSION

The effects of electrical stunning on catecholamine release indicate a degree of sympathetic stimulation not seen under any physiological circumstances including slaughter by bleeding. Catecholamine concentrations are several orders of magnitude greater after stunning in this manner than in sheep under moderate to severe environmental stimulation. The higher level of plasma adrenaline is in accordance with the predominance of this amine

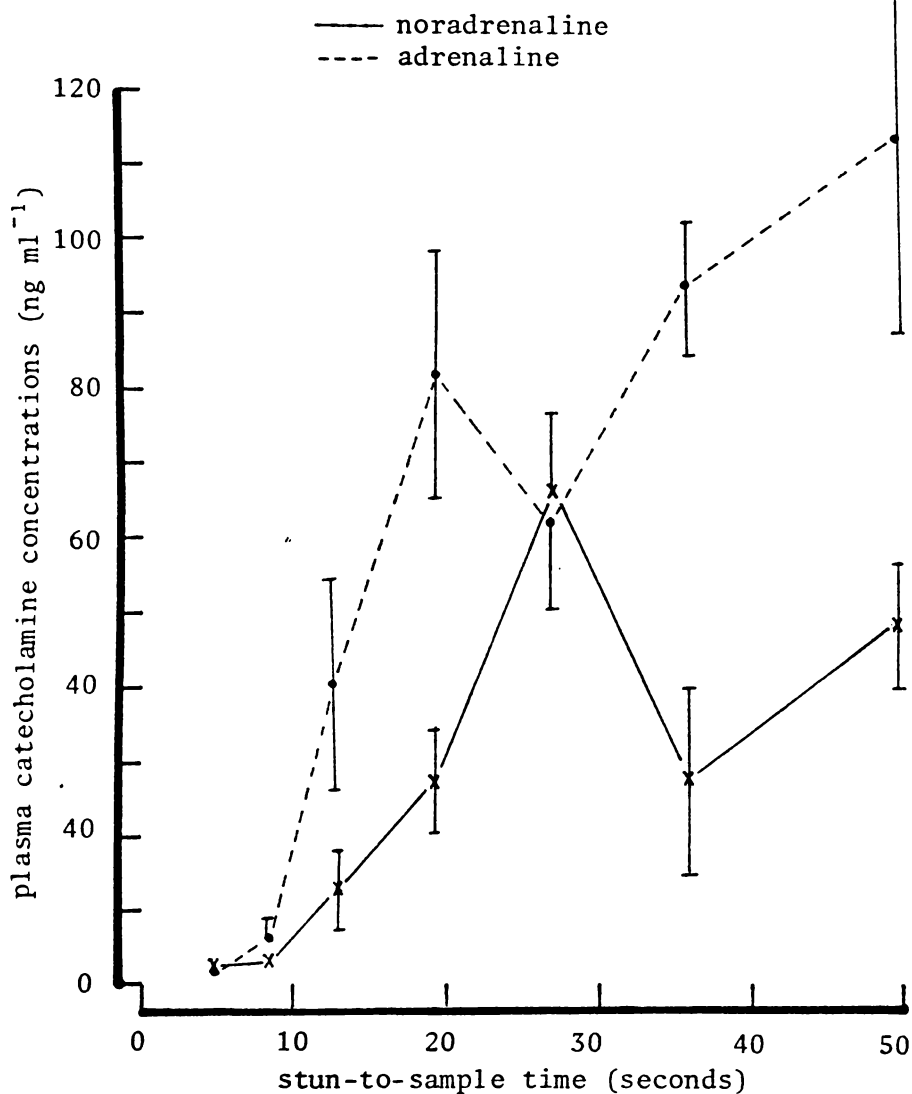


Figure 7-2. Catecholamine concentrations ($\pm$ SE) in the carotid plasma of electrically stunned and gash-cut lambs. Blood samples were collected at different times following stunning and the results pooled for each of the time intervals ≤ 5 , ≤ 10 , ≤ 15 , ≤ 20 , ≤ 30 , ≤ 40 , > 40 seconds.

Table 7-4

Mean Catecholamine and Cortisol Concentrations ($\pm$ SD) in Mobs of Slaughtered Lambs at a Large Abattoir

Mean Mob Splash Score	No. in Mob	Mob No. (Killing order)	No. Analyzed for Catecholamines	Noradrenaline (ng ml ⁻¹)	Adrenaline (ng ml ⁻¹)	No. Analyzed for Cortisol	Cortisol (ng ml ⁻¹)
0.44	27	4	10	57.3 $\pm$ 40.1	181.0 $\pm$ 59.8	11	48.8 $\pm$ 10.9
0.83	92	1	2	99.7	70.4	9	69.7 $\pm$ 21.1
0.97	29	2	4	31.9 $\pm$ 15.6	43.4 $\pm$ 9.9	11	69.8 $\pm$ 19.2
0.98	40	7	13	51.1 $\pm$ 11.1	83.6 $\pm$ 17.8	12	22.7 $\pm$ 6.0
1.06	141	3	26	71.6 $\pm$ 33.8	81.0 $\pm$ 28.3	13	77.8 $\pm$ 16.4
1.12	111	5	24	91.6 $\pm$ 43.9	94.3 $\pm$ 45.2	16	60.7 $\pm$ 20.9
1.46	588	6	109	75.7 $\pm$ 41.2	96.4 $\pm$ 51.8	42	73.1 $\pm$ 26.8
Mean concentrations				73.8 $\pm$ 40.0	96.9 $\pm$ 51.0		63.7 $\pm$ 26.3

analysis of variance of differences between mobs on logarithmically transformed data: cortisol $p < 0.001^{***}$, noradrenaline $p < 0.5^*$, adrenaline $p < 0.001^{***}$

Table 7-5
Cortisol and Catecholamine Concentrations ($\pm$ SD) in Blood Splash
Score Groups (Mob 6)

Blood Splash Score	No. Analyzed for Cortisol	Cortisol (ng ml ⁻¹)	No. Analyzed for Catecholamines	Nor- adrenaline (ng ml ⁻¹)	Adrenaline (ng ml ⁻¹)
0	10	80.6 $\pm$ 21.1	28	89.9 $\pm$ 52.2	101.1 $\pm$ 49.7
1	6	66.0 $\pm$ 33.4	26	68.6 $\pm$ 25.3	87.1 $\pm$ 33.6
2	10	67.2 $\pm$ 29.2	16	80.2 $\pm$ 24.1	99.2 $\pm$ 49.9
3	11	79.2 $\pm$ 31.0	23	58.3 $\pm$ 34.7	84.7 $\pm$ 40.2
4	5	65.0 $\pm$ 15.9	16	83.2 $\pm$ 53.9	117.4 $\pm$ 85.0

over noradrenaline in the adrenal medullary tissue of lambs. The proportion of adrenaline in the foetal lamb (Comline & Silver 1961) and foetal calf (Silver 1960) is small but increases rapidly during early postnatal life (Comline & Silver 1961). Adrenaline in the adrenal medullae of lambs 7-8 weeks old forms 69% of the total catecholamine (adrenaline plus noradrenaline) content with a similar ratio maintained in the catecholamines secreted in response to splanchnic stimulation (Comline & Silver 1961). However some of the noradrenaline measured in the blood of stunned lambs may be of sympathetic origin.

While there is a considerable element of individual and mob variation in the samples the overall means are higher than those reported in the electrically stunned pig (Althen *et al.* 1977). The adrenaline concentrations are similar to those found in rifle-stunned pigs but the level of noradrenaline in the lamb is very much lower (Althen *et al.* 1977). Perhaps this difference arises because the porcine adrenal contains a greater catecholamine concentration and a higher proportion of noradrenaline than the ovine adrenal (West 1955).

The apparent difference between catecholamine concentrations in the stunned lambs from the 2 slaughter locations may be mob-related or it may be an artefact dependent on the exact timing of the blood collection after stunning. Immediately after stunning the catecholamine content in the blood enters a state of rapid flux. Normally the blood circulation time in the lamb from the superior vena cava, through the right heart and the lungs and back through the left heart is of the order of 2 seconds (Reynolds *et al.* 1954). Stunning is likely to modify this time in view of the altered cardiovascular status (§7.6) but clearly the elapsed time from stunning is an important parameter in the plasma catecholamine concentration found (Figure 7-2). Because of the ready access to lambs at the small abattoir the blood collection in relation to the stunning was several seconds earlier than at the large abattoir. Other factors should not be overlooked. Prior stressors may condition nervous tissue to subsequent stimuli or there may have been differences in the effectiveness of the electrical stunning between the 2 sites. While the noradrenaline concentrations in the non-stunned lambs were similar at the 2 abattoirs the adrenaline levels were higher at the large abattoir (Table 7-3). In this case the difference cannot be attributed to differences in collection technique but is indicative of the greater environmental stressor level at the large abattoir compared to the small facility. Kilgour & de Langen (1970) came to a similar conclusion on the basis of cortisol levels in sheep also slaughtered at these abattoirs.

Taken overall however there is no evidence to support any causal effect or relationship between blood splash and plasma catecholamine levels in lambs after stunning. If such a relationship does exist it must be weak and obscured by the uncontrolled and supervening circumstances of the stunning and sampling procedures and the nature of the catecholamine release into the bloodstream.

7.5 THE ROLE OF PRESLAUGHTER STRESS IN THE AETIOLOGY OF BLOOD SPLASH

INTRODUCTION

It is apparent that some insight into the underlying physiological mechanisms of blood splash could be gained if the variation within and between different lamb mobs could be explained. The literature covering preslaughter stress in relation to carcass haemorrhage is confined to a few generalized observations with little documented data. As early as 1931 Tweed *et al.* observed that cattle, sheep and pigs that had travelled a long distance to the point of slaughter were more likely to have a higher incidence of blood splash than other animals. Animals that were difficult to restrain or had escaped and been chased were also claimed to be more susceptible. It was speculated that fear caused by noise, handling and restraint could have been a factor and that the effect was mediated either directly through some action of adrenaline or indirectly by causing excitement and muscular fatigue with consequent engorgement of muscle vasculature at the time of stunning. In Australia considerable variation in the incidence of blood splash is exhibited by different cattle mobs (Whittem *et al.* 1960). Seasonal and regional variations are also apparent. In a very limited survey (5 instances in a half-day of observations) no correlation was seen between abnormal handling stressors and blood splash. Amongst Queensland cattle Charles (1960) again observed a mob-dependent splash incidence. While supplying no supporting data he considered that blood splash in these cattle was "brought about by physical and mental stress applied to a susceptible animal at the time of slaughter". Avoidance of thermal stress by the use of covered yards was recommended. Hatton & Ratcliff (1973) could find no increase in the incidence of muscle haemorrhage in pigs agitated by the use of electric goads for 60 seconds immediately prior to stunning and slaughter. Recent results under New Zealand commercial conditions indicate that quiet handling of lambs prior to slaughter (without the use of dogs or rattles) has no effect on the blood splash levels compared with those handled under the usual conditions (Frazerhurst 1976).

On various physiological grounds it is not unreasonable to expect some link between a neuromuscular phenomenon and the state of animal arousal. The emergence of the blood splash lesion must be related to the sensitivity of muscle and/or nervous tissue at the time of stunning, but whether this is a significant variable remains unknown. The responses of mice and rats to electric shock have been used for some time to screen for anti-epileptic drugs. While such experiments may seem remote from blood splash in the lamb the results of a number of studies on the interaction of various stressors with physiological reactions to electric shock may throw some light on the data collected in the present investigation. The susceptibility of rats to muscular seizure in response to electric shock is age dependent. The younger the rat the more susceptible it is both in the immediate physiological reaction (Woodbury & Davenport 1952) and in subsequent brain development (Wasterlain & Plum 1973); this parallels the vulnerability of lambs compared with mature sheep in the emergence of blood splash (Frazerhurst 1976).

In mice the effect of up to 60 seconds restraint significantly increases their sensitivity to seizure, induced either by the drug pentylenetetrazol or by electroshock (Swinyard *et al.* 1962). The increased sensitivity was highest when the restraint was of a few seconds duration decreasing to a value close to that of control mice after 60 seconds of restraint. Evidently an adaptation occurred as the stress period was lengthened. Restraint did not alter the responses of adrenalectomized rats while adrenaline (0.0312 and $0.125 \mu\text{g g}^{-1}$ iv) increased the sensitivity. Finally the change in the threshold to electric shock varied with the length of time after cessation of restraint. A maximum plateau of sensitivity was reached 30 seconds after restraint. Pain and postural disequilibrium induced similar effects (Swinyard *et al.* 1963).

With rats 10 minutes of forced fighting raises the electroshock seizure threshold (Woodbury & Davenport 1952) as does severe trauma resulting from placing rats in a revolving drum (42 revolutions min^{-1}) (Goldberg & Salama 1969). In the latter experiment a 2-fold increase in the time to seizure was seen immediately after the application of the stressor. This was followed by a return to control values if the shock was applied 10-15 minutes later. After 15 minutes a secondary increase in the average time to seizure occurred and lasted for several hours. Adrenalectomy or administered cortisol and deoxycorticosterone made no difference to the observed behaviour but reserpine, a depletor of catecholamine stores in adrenergic structures, antagonized both phases of the increased latency seen after the stressor. With chickens similar complex time and stressor intensity relationships in the electroshock response have been documented (Nash & Gallup 1975). Other factors which effect electroshock thresholds are temperature (Woodbury & Davenport 1952) and nutrition (Davenport & Davenport 1948). The electroshock threshold to seizure in rats is lowered when dietary intake is restricted but returns to control level on reversion to a normal intake (Davenport & Davenport 1948).

There is reason to expect also that the arterial blood pressure at the moment of stunning may effect the subsequent pressor response (§7.6) (Korol & Brown 1967) and many environmental stimuli in the experience of slaughter stock will influence arterial blood pressure both acutely and chronically.

Taken together this evidence suggests that if "stress" is a factor in the aetiology of blood splash then its influence may be a complex amalgam of the type, duration, intensity and timing of the stress in relation to stunning.

METHODS

Experiment 1 Seven mobs were studied at a large freezing works, scored for blood splash and blood samples collected as described in §7.4. The sample of mobs selected from those arriving at the abattoir was as representative as possible of one day's killing. The size of the mobs varied from 27 to 588, the distances travelled ranged from 16 to 225 km from Taumarunui in the south to Whakatane in the east, and the lambs were on the trucks from 1 to 8 hours.

Table 7-6 sets out the on-farm factors considered to have some relationship with preconditioning to the stressors of transport and abattoir handling. Each of these variables was given an arbitrary score on a 1-3 rating scale (1 for the most preconditioned ranging to 3 for the least) except in the case of dogs, a factor common to all 7 farms. As 2 mobs had been shorn these were given a lower score than the woolly mobs which would be under greater thermal stress during travelling and handling in summer temperatures. The farms were also rated on the size of paddock subdivision (≤ 5 ha, ≤ 10 ha, > 10 ha), since smaller paddocks mean that lambs on such properties probably experience more handling than those on larger ranges. The farms which used a motor bike rather than a horse were given a lower rating as such lambs would have had some introduction to mechanical and traffic noises. Farm topography was roughly divided into 3 groups: flat, hilly and steep. It was considered that lambs from flat areas had less exercise and consequently lower muscle tone and was speculated that these lambs would have a less violent muscular reaction to stunning than those used to movement on steep hill country. After assigning the rating to each factor, the total for all factors was used as a method of ranking each mob for background experience emanating from the farm. This score was one of several used to give an overall rating of stressor exposure in the history of the individual lamb mobs.

Table 7-6

On-farm Factors Relating to the Mobs Slaughtered

Killing Order	Mean Mob Splash	No. in Mob	Hours on Truck	Distance Travelled (km)	Woolly/Shorn	Ave. Size of Paddock (ha)	Dogs Used Yes/No	Farm Bike Yes/No	Weaned Yes/No	Breed*	Type of Country
1	0.83	92	6	160	W	8	Y	N	Y	S x R	hilly
2	0.97	29	2	88	W	8	Y	Y	Y	R x S; x BL	hilly
3	1.06	141	2¼	100	W	8	Y	Y	Y	R x S; x BL	hilly
4	0.44	27	1¼	40	W	3½	Y	N	N	S x R	flat
5	1.12	111	8	225	W	4	Y	Y	Y	Su x Coop	easy
6	1.46	588	3½	188	S	25	Y	N	Y	R	easy to steep
7	0.98	40	¾	16	S	8	Y	N	Y	S x R	rolling

* S Southdown, R Romney, BL Border Leicester, Su Suffolk, Coop Coopworth

Another factor rated was mob size on the grounds that the bigger the mob the greater the chance of being mixed with unfamiliar flock mates and the longer the various handling procedures would take to complete. Duration of travel, killing order and general excitement at the point of killing as determined by the cortisol analysis were also ranked. Killing order is weakly related to blood splash incidence with a tendency for the severity to increase during the course of a day (unpublished data). Catecholamine levels were excluded because they are overwhelmingly influenced by the stunning itself.

A number of lambs which were subjected to a variety of short-term handling stressors were marked for later identification. Stressors included being trampled during trucking, being roughly handled by man or dog, inhalation of water and near drowning in the sheep wash, and being held for upwards of 10 minutes in the restraining conveyor during breaks in killing.

Experiment 2 At a later date the same information was collected (although without the cortisol analyses) regarding a further 8 mobs of lambs.

RESULTS

Experiment 1 A wide range of mean blood splash scores from 0.44 to 1.46 was associated with the different mobs. When the individual stressor factors were compared to the splash severity (Spearman's rank correlation coefficient) only mob size was significantly ($p < 0.05$) correlated (Table 7-7). When the individual factors were combined into an overall index this too was significantly related to the splash scores ($p < 0.05$). Five of the mobs had a similar mean splash score (between 0.83 and 1.12) with one mob much lower (0.44) and another much higher (1.46). The combined index clearly separated these 3 groups.

Table 7-7

Ranking Order of Stress Parameters with Blood Splash Incidence

Mean Blood Splash	Killing Order	Duration of Travel	Number in Mob (*)	Previous On- farm Ranking	General Excitement (Cortisol)	Overall Mean Score	Overall Mean Rank (*)
1 (0.44)	4	2	1 (27)	2	2	2.2	1
2 (0.83)	1	6	3	6	4	4.0	4=
**3 (0.97)	2	3	2	3	5	3.0	2
4 (0.98)	7	1 ($\frac{3}{4}$ hr)	6	5	1 (22.7)	4.0	4=
**5 (1.06)	3	4	5	4	7 (77.8)	4.6	6
6 (1.12)	5	7 (8 hr)	4	1	3	4.0	4=
7 (1.46)	6	5	7 (588)	7	6	6.2	7

lowest and highest values for some factors given in parentheses

* significantly correlated with mean blood splash ranks ($p < 0.05$) (Spearman's rank correlation)

** lambs from 2 farms run in common by father and son

Fifteen lambs were identified as being subjected to abnormally severe stressors either on the day of arrival or during the subsequent day prior to killing. A further 12 lambs were held up at the head of the constraining conveyor during delays in killing. The mean blood splash levels of these lambs did not however differ significantly from the other lambs (Table 7-8) although the small number of lambs in each category may obscure any weak relationships.

Table 7-8
Blood Splash in Lambs Associated with
Specific Stressors

	n	Mean Blood Splash $\pm$ SD
day of arrival	8	1.25 $\pm$ 1.28
day of killing	7	1.57 $\pm$ 1.51
killing breaks	12	1.67 $\pm$ 1.50
mean splash (of all lambs)	1028	1.23 $\pm$ 1.28

Experiment 2 A second experiment to test the predictive power of the overall stressor index was carried out but unfortunately on this occasion all the mobs had a low and relatively uniform blood splash incidence (between 0.16 and 0.42). None of the individual factors nor the overall index (which did not however include cortisol levels) was significantly correlated with the blood splash.

CONCLUSIONS

The data gathered in the present investigation are limited but confirm other recent conclusions (Frazerhurst 1976, Kirton & Frazerhurst 1976) that short term stressors at the abattoir do not appear to be a major factor in predisposition to blood splash. The results of the initial survey on the total lamb history and its relationship to blood splash are interesting but

do not warrant a definite conclusion at this stage. Many more mobs should be examined representative of a diverse range of backgrounds and exhibiting a wide spread in the level of blood splash. It seems improbable however from the present results that environmental stressors and preconditioning to handling are the sole sources of the variability in the susceptibility of mobs to blood splash.

7.6 PHYSIOLOGICAL MECHANISMS IN THE DEVELOPMENT OF THE BLOOD SPLASH LESION

INTRODUCTION

While research into the physiological origin of blood splash has been hampered by the sporadic incidence of the condition and by the lack of a suitable experimental model, the data accumulated to date suggest that blood splash results directly from the severe muscular spasm induced by stunning combined with an increased blood pressure shortly afterwards. Light and electron microscopic studies in mice (Shaw *et al.* 1971) and lambs (Leet *et al.* 1977) of areas about ruptured blood vessels have shown a markedly distorted muscle structure. A phenomenon known as "supercontracture" may be responsible for the blood splash lesion with extreme muscle fibre contraction and even separation of adjacent fibres from each other (Leet *et al.* 1977). It is postulated that blood vessels in proximity to such areas of violent contraction are ruptured leading to a loss of blood to surrounding tissue. Neuromuscular blocking drugs as gallamine are effective in suppressing the development of blood splash (Leet *et al.* 1977).

Electrical stunning results also in marked increases in arterial blood pressure (Clark & Tweed 1932, Mandrup 1964, Kirton *et al.* 1978). In intact lambs the rise is of the order of 3.5 (2.9-4.1) times baseline with a maximum reached at 11 ± 3.4 seconds after stunning and a recovery time of 115 ± 25 seconds (Kirton

et al. 1978). Even in lambs gash cut 5-8 seconds following stunning, transitory blood pressure rises close to the maximum expected in intact animals may be anticipated. Increased blood pressure alone is not the cause of blood splash as it may still occur in lambs stunned after the neck blood vessels have been severed - effectively preventing the arterial blood pressure rise (Mandrup 1964, Kirton *et al.* 1978). But this procedure lowers the blood splash incidence, as does gash-cutting immediately after stunning (compared with the usual delay of 5-8 seconds) (Kirton *et al.* 1978). Thus while blood pressure alone is unlikely to constitute the major factor in the aetiology of blood splash it may be expected to exacerbate the condition both by forcing additional quantities of blood from the circulation through vascular ruptures and perhaps also by breaking already weakened points in these walls (Kirton *et al.* 1978).

Catecholamine involvement in blood splash development seems likely if only because of their importance in cardiovascular control (§5.2) and the demonstration of greatly increased sympathetic activity associated with stunning (§7.3). However there are also some data describing significant effects of the α and β receptor blocking drugs propranolol and phentolamine on lesion development in rats (Shaw *et al.* 1971). Propranolol ($\sim 1.2 \text{ mg kg}^{-1}$, ip) resulted in a reduction in the haemorrhage extent ($p < 0.05$) while phentolamine ($\sim 11.8 \text{ mg kg}^{-1}$, ip) increased the extent ($p < 0.01$). The authors postulated that propranolol caused vasoconstriction by an indirect stimulation of α receptors while phentolamine caused vasodilation owing to blockade of α -mediated constriction. The suggested consequences of the resulting changes in arterial flow were a decrease and an increase respectively in the capillary pressure and hence the observed changes in the levels of blood splash.

During electroshock in dogs asystole with no discernible blood pressure persists as long as the current continues. Afterwards a slow rate of heart beat resumes followed by a marked increase in blood pressure and an increase in plasma catecholamines (Anton

et al. 1977). Premedication with the α blocker phenoxybenzamine (1.5 mg kg^{-1} at -40 minutes) prevented the bradycardia, the blood pressure rise and the plasma adrenaline response. Propranolol (2.5 mg kg^{-1} at -15 minutes) was without effect on these parameters. The catecholamines released by electroshock appeared to be principally responsible for the pressure rise since it was abolished by spinal sympathetic block or phenoxybenzamine. Furthermore the ganglionic blocking drug chlorisondamine which prevented the rise in circulating noradrenaline (but not of adrenaline) moderated the peak blood pressure (Anton *et al.* 1977).

The present experiment was designed to examine the effect of propranolol and phentolamine on blood splash incidence in electrically stunned lambs.

METHODS

A group of 103 lambs of mixed breed and sex was trucked to the abattoir the day before the experiment. The lambs were stunned with a Stampco electrical stunner (Stampco Engineering Pty Ltd, Carlton South, Victoria, Australia) and slaughtered by spear sticking (severance of the carotid and jugular vessels by a lateral stab incision while preserving the trachea and oesophagus intact).

Eighty seven of these lambs (mean weight $24.6 \pm 3.6 \text{ kg}$) were divided into 4 treatment groups.

1. *Non-Restrained Controls* No drugs or saline were administered to this group and handling prior to stunning was minimal.
2. *Saline Controls* Approximately 15 ml of saline was administered over a period of 1 minute into the jugular vein with restraint of the animal. It was then kept in a holding pen for exactly 10 minutes from the end of the injection before stunning and slaughter.

3. *Phentolamine Treated* (Rogitine, Ciba Laboratories, Horsham, UK.) 1 mg kg⁻¹ body weight of phentolamine in a solution of 1 mg ml⁻¹ was administered over a period of exactly 1 minute into the jugular vein and the animal kept for 10 minutes before stunning and slaughter.
4. *Propranolol Treated* (Inderal, ICI Ltd, Macclesfield, UK.) 1 mg kg⁻¹ body weight of propranolol in a solution of 1 mg ml⁻¹ was administered over a period of exactly 1 minute into the jugular vein and the animal kept for 10 minutes before stunning and slaughter.

Lambs were slaughtered in groups of 3 consisting of a control (either group 1 or group 2) and one of each drug class. Two other larger groups of controls only were slaughtered together. Lambs were assessed for blood splash on the 0-4 scale described in §7.4. Scores were recorded for the heart exterior surface, the gall bladder and the carcass. The heart interior surface was also examined and scored in the latter half of the experiment. As blood vessels appeared to be dilated in some early carcasses examined (thought to be probably associated with the administration of vasoactive drugs) the gall bladder was scored for the appearance of surface vessels.

RESULTS

No obvious differences in the behaviour of the drugged and non-drugged lambs was apparent; nor was there any observable difference in the reaction to electrical stunning. Inspection of the results of the scoring for gall bladder vessel dilation showed no apparent relation between the degree of dilation and any particular treatment group. The degree of carcass splash overall was very low and of no commercial significance. Apart from 2 carcasses scoring 2 and 3 all remaining carcasses were assessed as 0 or 1. Both phentolamine and propranolol suppressed the development of blood splash (Tables 7-9, 7-10).

Table 7-9

Mean and Total Accumulated Blood Splash Scores

Group	Gall Bladder	Exterior Heart	Interior Heart	Carcass	Mean and (Total)
1 (n=21)	0.67 (14)	0.29 (6)	1.10 (11)	0.29 (6)	0.59 (37)
2 (n=20)	0.75 (15)	0.40 (8)	0.55 (11)	0.30 (6)	0.50 (40)
3 (n=23)	0.09 (2)	0.00 (0)	0.07 (1)	0.00 (0)	0.04 (3)
4 (n=23)	0.26 (6)*	0.09 (2)	0.00 (0)	0.05 (1)	0.10 (9)

accumulated splash scores in parentheses

* includes a gall bladder score of 4

NB The number of observations varies slightly for each group of assessments and the number of internal heart observations was confined to the last 59 lambs.

Table 7-10

Percentage of Carcasses Showing Some Degree of Blood Splash
(ie. a score of 1 or more)

Group	Gall Bladder	External Heart	Internal Heart	Carcass	Mean
1	43	24	60	19	37
2	35	35	45	35	38
3	4	0	7	0	3
4	9	9	0	4	6
	**	**	***	**	
1 + 2	39	29	50	27	37
3 + 4	7	4	4	2	4
	***	**	***	***	

** $p < 0.01$

*** $p < 0.001$

(chi-square analysis of differences between treatments)

Overall there were statistically significant differences amongst the treatment groups but no differences between the 2 control groups (groups 1 and 2) or between the drug treatment groups (groups 3 and 4). Thus the procedure involved in administering the drug did not appear to influence the development of blood splash. When the data from the control lambs are pooled and compared to the pooled treated lambs the difference becomes significant at the 0.1% level for all carcass areas examined except the external heart ($p < 0.01$) (Table 7-10).

Blood Pressure Experiment

Using the methods described in §5.2 the arterial blood pressure and heart rate were determined in 4 sheep 10 minutes before and 60 minutes following the intravenous administration (over 1 minute) of either phentolamine (1 mg kg^{-1}) or propranolol (1 mg kg^{-1}). No effect of propranolol on either heart rate or blood pressure emerged but phentolamine markedly increased the heart rate and reduced blood pressure by an average of 23 mm Hg with a nadir reached at about 5 minutes (Figure 7-3). At 10 minutes blood pressure was still significantly reduced (by 16 mm Hg on average) full recovery taking longer than 60 minutes in 3 of the 4 sheep.

DISCUSSION

To propose a mechanism for the reduction in blood splash effected by propranolol and phentolamine we must examine the actions of these substances on neural and cardiovascular function. Unlike the results reported in the rat where propranolol reduced and phentolamine increased splash incidence (Shaw *et al.* 1971) any explanation in the present case must encompass α and β mechanisms that promote or facilitate the development of this lesion. Difficulties occur in the application of literature results because of differences in the route of administration, because different α and β antagonists vary in their persistence, are usually not completely selective for one receptor subtype and because of the physiological interrelationships existing between some responses.

7.6 $1 \text{ mg kg}^{-1} \text{ (iv)}$
phentolamine

324

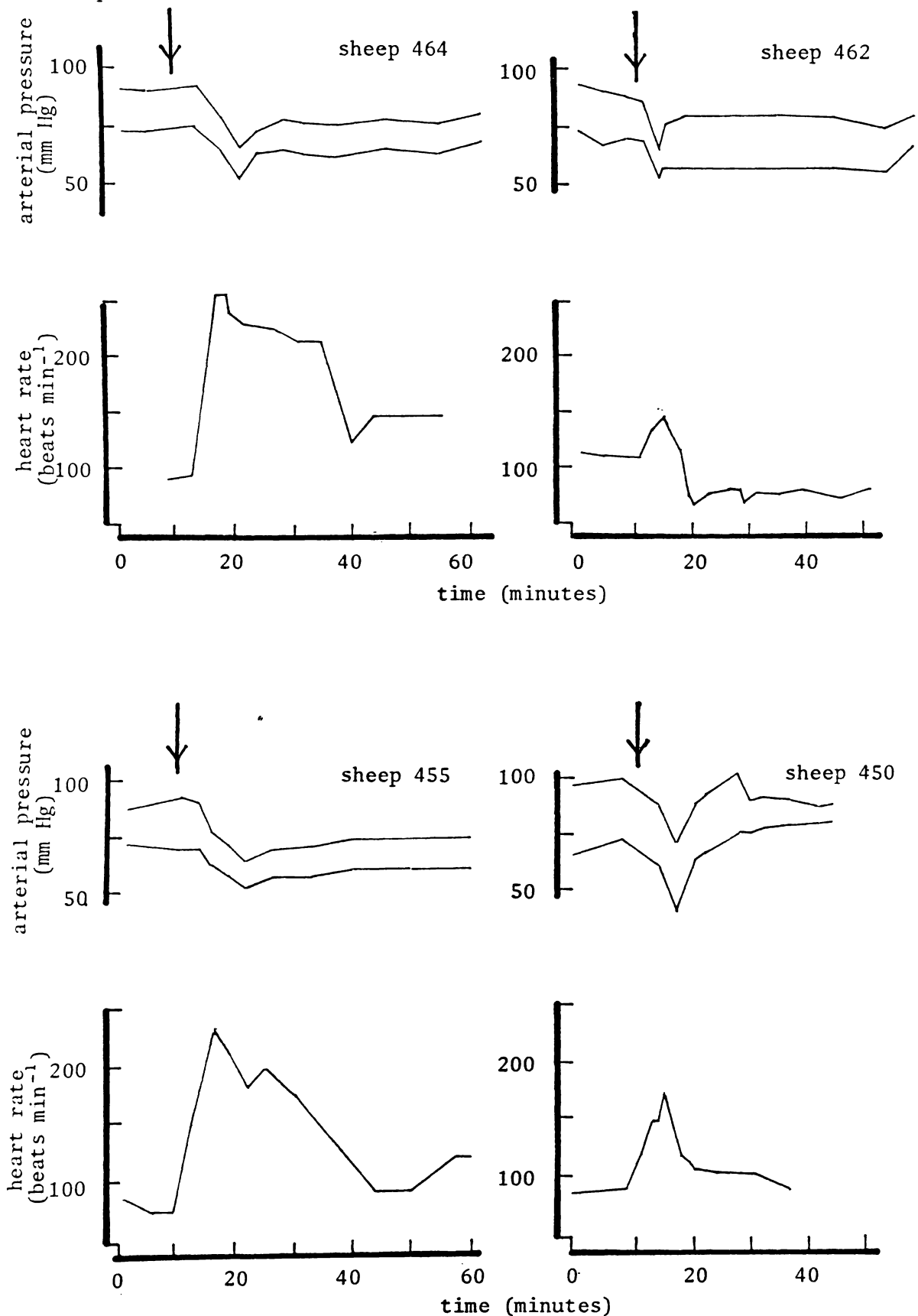


Figure 7-3. Arterial blood pressure and heart rate responses of sheep to 1 mg kg^{-1} of phentolamine (iv).

Since both the adrenergic blockers used in this experiment may be classed as hypotensives it seems logical to look at their effects in reducing blood pressure in the resting condition and at their possible effects in attenuating the acute blood pressure rise associated with electrical stunning. Propranolol has come into widespread clinical use as an antihypertensive agent over the last decade but its mode of action in reducing blood pressure remains a matter of dispute. In healthy individuals it is effective in reducing the increases in the heart rate in response to mental stress, catecholamines and exercise (Taylor & Meeran 1973). Four principal actions have been identified: (1) peripheral β blocking effects on the vasculature and the heart (2) a central hypotensive effect (3) interference with neural or neuromuscular transmission by mechanisms that may be unrelated to its β receptor blocking properties (4) suppression of renin release. Propranolol may however produce an acute rise in blood pressure on intravenous injection in the rat due to blockade of β receptor vasodilation especially in the presence of α agonists (Regoli *et al.* 1972, Yamamoto & Sekiya 1972). In general as in the 4 sheep examined, acute intravenous administration of propranolol does not reduce resting blood pressure but does attenuate pressure rises due to various stimuli (Tarazi *et al.* 1976) although the antihypertensive effect in humans is generally only seen after prolonged administration of the drug. A variety of evidence strongly suggests the existence of both α - and β -adrenergic receptors in the brain modulating arterial blood pressure both chronically and acutely (Day & Roach 1974). Propranolol readily crosses the blood-brain barrier and it is likely that blockade of central β receptors is an important component of the action of this drug (Dollery & Lewis 1976). Propranolol prevents the increase in renin secretion caused by various stimuli including the catecholamines (Assaykeen *et al.* 1970) and it has been argued that the clinical usefulness of β blockers in some forms of hypertension arises from this effect (Weber *et al.* 1975). Nevertheless the compressed time scale of events in the development of blood splash would rule out involvement of this particular mechanism.

Propranolol has been shown to inhibit transmission in adrenergic sympathetic neurones in isolated smooth muscle preparations via a presynaptic mechanism (Day *et al.* 1968) as well as inhibiting tetanic contractions in rabbit muscle in response to high frequency stimulation (Myhre *et al.* 1977). Furthermore propranolol can displace noradrenaline from adrenergic nerve endings at rest and has been shown to inhibit the stimulation-induced release of neurotransmitter by a mechanism independent of β -adrenergic receptor blockade or local anaesthetic effects, thus inhibiting muscle responses to electrical stimulation (Saelens *et al.* 1977). While propranolol-inhibition of muscle response and particularly of supercontraction due to electrical stunning is an attractive explanation of the present results, the inhibition of peripheral sympathetic activity is thought to develop slowly as propranolol accumulates within the nerve endings (Saelens *et al.* 1977). The acute administration of propranolol to dogs has been reported to have no effect on neurotransmission evaluated *in vivo* (Dawes & Faulkner 1975). Furthermore this mechanism does not account for the bulk of muscles not directly innervated by the sympathetic nervous system.

The direct actions of catecholamines on skeletal muscles are complicated because they vary with the muscle type and condition, are species dependent and the physiological significance of the observations to date are difficult to assess (Bowman & Nott 1969, Tomita 1975). In general however the effects of catecholamines in increasing fast-contracting skeletal muscle twitch tension are blocked by β antagonists. Neuromuscular transmission is accelerated by adrenaline when the stimulus is strong and this effect may be blocked by α antagonists (Bowman & Nott 1969, Tomita 1975). Propranolol has also recently been reported to depress muscle fibre action potentials in neuromuscular transmission (Larsen 1978).

The most obvious effect of phentolamine is to inhibit α -vaso-constrictor responses but it also has a non-specific dilator action on precapillary sphincters reducing vascular resistance (Takeshita *et al.* 1976). The acute reduction in baseline blood pressure itself is likely to reduce splash severity.

Phenoxybenzamine has been shown to prevent the rise in blood pressure associated with electroshock in the dog (Anton *et al.* 1977). Yet another possible mechanism by which α -adrenergic blockade could interfere with vasomotor effector systems in the electrically stunned lamb is via the spinal catecholamine-containing neurones which relay information from the central nervous system to the periphery via an α -adrenergic mechanism (Taylor & Brody 1976).

Clearly the possible mechanisms by which these drugs may exert their effects are numerous and complex and there are not enough data to make possible a choice between the possibilities. A further experiment to examine the probable protective effects of adrenergic blocking drugs against the extreme hypertension developed after electrical stunning of lambs (Kirton *et al.* 1978) is the logical next step in this investigation. The rat model of blood splash advocated by Shaw *et al.* (1971) for study of the effect in domestic stock appears unsuitable in view of the difference in the reaction between rats and lambs to phentolamine.

7.7 CONCLUSIONS

Clearly the electrical stunning of lambs is a major autonomic insult of which blood splash and catecholamine release are both manifestations. In fact a distinction should be drawn between the psychological humaneness of stunning, an aspect which has not been addressed in this work, and the physiological trauma which stunning represents. It has also become evident that the few consequences of electrical stunning which have been investigated to date (including metabolic effects, blood splash and the pressor response) all have an important catecholaminergic component. No attempt has been made to relate these findings to the ultimate meat quality but in view of the important metabolic effects of the catecholamines on postmortem biochemical processes there is a need to develop research in this area.

ASPECTS OF THE CATECHOLAMINES IN THE CENTRAL NERVOUS SYSTEM OF SHEEP

CHAPTER 8

8.1 INTRODUCTION

To this point the work described has encompassed predominantly the peripheral actions and the release of catecholamines. In recent years the discovery and mapping of dopaminergic and noradrenergic tracts in the central nervous system (§1.3) has formed the basis of a remarkable expansion in understanding the mechanism of brain functions and the manner in which many drugs exert their action. These data are far from complete and it may well be that many present concepts will undergo future radical change.

The investigations described in the present chapter arose from the hypothesis that the motor symptoms of the sheep disorder called "ryegrass staggers" could result from a disruption of central catecholamine neurochemistry in brain centres controlling locomotion and posture. In humans the most common lesion of these centres is caused by Parkinson's disease, in which the symptoms are not unlike those observed in ryegrass staggers. Some parallels are drawn between the two syndromes, preliminary trials to test the hypothesis are described and some of the behavioural effects of the central dopaminergic blocking drug haloperidol in sheep examined.

8.2 RYEGRASS STAGGERS AND PARKINSON'S DISEASE

Ryegrass staggers is a common neuromuscular disorder affecting grazing animals in New Zealand and Australia, the name arising from the staggering gait symptomatic of the disorder and its association with ryegrass-dominant pastures (Byford 1978). Outbreaks are mainly seen in summer and autumn especially during or shortly after long dry spells and affect sheep, cattle and horses. The main symptoms are tremors either confined to the head or extending over the whole body, motor inco-ordination, akinesia and in severe cases temporary collapse. Affected sheep may manifest few outward signs when standing at pasture but develop inco-ordination and muscular spasms when attempting to move on being disturbed. Recovery occurs within 1-2 weeks of transfer to safe pasture (Byford 1978). Although long term effects are absent ryegrass staggers exacts an economic toll in deaths through accidents (drownings, falling over cliffs) in difficult mustering and in the necessity to spell toxic pasture.

The cause is at present still not defined though a mycotoxin of either soil (di Menna & Mantle 1978) or ryegrass litter (Latch *et al.* 1976) origin is suspected. Tremorgenic strains of *Penicillia* known to be present in New Zealand soil have been isolated from the faeces of affected sheep (di Menna & Mantle 1978) and fungal tremorgens have been shown to produce typical ryegrass staggers symptoms (Gallagher *et al.* 1977). Peripheral lactic acid levels are increased in calves after dosing with a tremorgenic mycotoxin and total peripheral catecholamine levels show an approximately 8-fold increase 2-4 hours after such dosing (Cysewski *et al.* 1975). Almost nothing is known of possible neurological lesions involved in ryegrass staggers; no neurochemical studies have been published and the reported degeneration of cerebellar Purkinje cells has been dismissed as a non-specific secondary consequence of the disease (Mason 1968).

If the condition is central in origin, as is likely, some disturbance to central nervous system neurotransmitter physiology might be expected, and especially in the cerebral motor cortex, the basal ganglia and the cerebellum which have an important function in motor control (Evarts 1974). Dopamine is the principal neurotransmitter in the basal ganglia associated with the control of locomotion (Lloyd & Hornykiewicz 1975).

Abnormally low basal ganglia tissue levels of dopamine and lesser losses in noradrenaline and serotonin are found in diseases affecting motor function in humans such as Parkinson's disease (Hornykiewicz 1973). Parkinson's disease is a chronic and progressive degenerative disease of the central nervous system with clinical features that can be reduced to 3 cardinal symptoms: tremors which cease when the affected limb is moved voluntarily but return as soon as the movement comes to an end, rigidity or stiffness of the skeletal muscle and akinesia or difficulty in initiating voluntary movements or modifying on-going motor activity.

It is thought that the lack of dopamine in the brain of Parkinson's patients and the consequent disruption in the transmission of nerve impulses is the cause of these symptoms. Low levels of dopamine in neural tissue are also reflected in the brain and cerebrospinal fluid as low levels of the principal metabolite 3-methoxy-4-hydroxyphenylacetic acid (homovanillic acid) (Gottfries *et al.* 1969, Parkes *et al.* 1971). Homovanillic acid in the cerebrospinal fluid is derived from the brain and shows a progressive decrease in concentration as the cerebrospinal fluid flows from the lateral into the third and fourth ventricles, the cisterna magna and down the spinal cord - reflecting reabsorption by an active transport process (Gordon *et al.* 1975). Thus changes in brain dopamine turnover are associated with changes in cerebrospinal homovanillic acid in the same direction (Curzon 1975). However Curzon (1975) also points out that central dopaminergic activity is not the only factor that affects cerebrospinal fluid concentrations. Other effects such as changes in the partitioning of homovanillic acid between

different routes of elimination from the brain, the rates of transport from the cerebrospinal fluid to the blood and from the lateral ventricle to the lumbar sac, the cerebrospinal fluid volume and methodologic problems are significant factors in the interpretation of cerebrospinal fluid homovanillic acid content (Curzon 1975, Moleman *et al.* 1978).

The central dopaminergic system may be functionally enhanced by agents that increase the synthesis of dopamine (such as l-dopa) delay the catabolism of dopamine (monoamine oxidase inhibitors), stimulate its release or its action at receptor sites and block the reuptake of monoamines (drugs of the amphetamine class). Oral l-dopa with or without a decarboxylase inhibitor (to restrict peripheral metabolism) is the present most effective agent in the treatment of Parkinson's disease, though the exact mechanism of action remains obscure and its efficacy declines with long term use (Marx 1979). L-dopa, unlike dopamine, is able to cross the blood-brain barrier (§5.5) where it is decarboxylated to dopamine. To this extent l-dopa treatment may be regarded as dopamine replacement therapy. L-dopa treated patients have elevated brain concentrations of dopamine and homovanillic acid, greatly in excess of dopamine levels from non-treated patients and these may even exceed levels in normal subjects (Hornykiewicz 1973, Rinne & Sonninen 1973). However from a biochemical and pharmacological point of view l-dopa is not an ideal drug because of its short half life (§5.3). It is quickly and extensively metabolized in the peripheral tissues so that rather high and frequent doses are necessary in order to obtain effective symptom relief. These high doses are often associated with undesirable side effects related to high circulating dopaminé levels (Klawans & Bergen 1975).

Anxiety or emotional tension increases the symptom severity in Parkinson's disease as does intravenous adrenaline (Barcroft *et al.* 1952, Conostas 1962, Owen & Marsden 1965). The increase in tremor in response to adrenaline may be abolished by β -adrenergic blockers such as pronethalol and propranolol (Owen & Marsden

1965). Some protection against Parkinsonian tremor, though with considerable inter-subject variation, has been found with β blockers in some studies (Herring 1964, Owen & Marsden 1965) but not in the study of Vas (1966). Propranolol is widely prescribed in medicine as an anxiolytic (Suzman 1976).

Testosterone levels in rams affected by ryegrass staggers are abnormally low (Peterson *et al.* 1978). These authors suggested in explanation that monoamine oxidase might be inhibited by alkaloids known to be present in ryegrass, resulting in an increase in serotonin and noradrenaline content of the pineal and thence to an increase in melatonin and a decrease in gonadotrophin (luteinizing hormone and follicle stimulating hormone) release. However a decrease in peripheral testosterone level is also consistent with depression of dopaminergic transmission. Dopamine introduced directly into the third ventricle of the rat increases testosterone release (Naumenko *et al.* 1975) while dopaminergic blockage with haloperidol or chlorpromazine strongly inhibits gonadotrophin release in rats (Dickerman *et al.* 1974) and monkeys (Bhattacharya *et al.* 1972) and would also be expected to depress circulating testosterone.

Thus the motor abnormalities of ryegrass staggers and Parkinson's disease and symptom association with arousal or disturbance are in some respects similar. The parallel cannot however be drawn too far since Parkinson's disease is an irreversible degenerative lesion while complete recovery from ryegrass staggers occurs on non-toxic pasture. Nevertheless it could be hypothesized that in ryegrass staggers a toxin, probably of fungal origin, interferes with the synthesis, storage, uptake or receptor interaction of dopamine resulting in temporary functional impairment of motor centres. Several tests were undertaken against this background to examine the possibility of catecholamine involvement in ryegrass staggers.

EXPERIMENTAL METHODS AND RESULTS

Adrenaline Injections

Five sheep affected with ryegrass staggers were injected with adrenaline (1 mg kg^{-1} , sc). The degree of tremor was assessed subjectively over the 30 minutes postinjection but adrenaline in the dose employed was without effect.

Propranolol Injections

Five affected sheep were injected intravenously with the β blocker propranolol (1 mg kg^{-1}). The sheep were tested for effects on staggers severity by driving them before dogs and measuring the distance covered before the development of symptoms. A test was made 10 minutes before injection, 10 minutes after injection and at hourly intervals thereafter for 3 hours. Propranolol too was without noticeable effect in these tests.

Haloperidol Injections

As dopaminergic blocking drugs (including haloperidol) in sufficient doses cause the development of Parkinson-like symptoms in human subjects (Calne 1970, Crowley *et al.* 1976) a number of sheep were injected intravenously with haloperidol (in 1% lactic acid) at doses of 0.5, 1.0, 1.5 and 2.0 mg kg^{-1} on 5 separate occasions. Of the 30 sheep examined only 2 (injected with doses of 1.5 and 2.0 mg kg^{-1}) could be said to exhibit the typical stiff-legged "stagger" seen in ryegrass staggers, and then for a few minutes only. At doses above 1 mg kg^{-1} all sheep showed locomotive disturbances of various types, catalepsy and stereotyped ingestive behaviour (§8.3).

L-dopa Injections

Two sheep housed in covered pens developed ryegrass staggers after feeding for 3 weeks on toxic pasture. Each sheep was injected with 100 mg of l-dopa intravenously every 2 hours over a 24 hour period commencing on day 1 at 8.40 am. Since the trial was undertaken in winter and the amount of stored toxic pasture

available was limited, control saline-injected sheep were not available for testing with the 1-dopa-treated sheep. Assessment for staggers was made as in the propranolol injections; before the first injection of 1-dopa and then 10 minutes after each injection during daylight hours with another test on the morning of day 2. Further tests were carried out on days 3 and 4 in the early morning as well as a series of tests on day 5 after saline injections at 2 hourly intervals.

An improvement was noted in the time to onset of staggers during the first day of testing in sheep A (Figure 8-1a). In sheep B which was less affected by staggers the same improvement occurred and in fact after the first few tests it was able to complete the course without relapse. However after continuing the 1-dopa injections overnight the subsequent morning test showed a decline in the distance covered by both sheep. Further decreases occurred over the next 2 mornings (days 3 and 4). On day 5 with saline injections at 2 hour intervals an improvement again occurred with repeated testing, though of lesser magnitude than during day 1.

Cerebrospinal Fluid Homovanillic Acid

Ryegrass staggers-affected sheep and healthy sheep from clover pasture were sampled for cerebrospinal fluid from the cisterna magna and the samples analysed for homovanillic acid (§4.6) and lactic acid (§4.7). To obtain cerebrospinal fluid a cisternal puncture was performed in lightly anaesthetized sheep (12-15 ml of Nembutal sodium (60 mg ml⁻¹) Abbott Laboratories, Chicago, Illinois, USA). A syringe needle (20 gauge) was introduced in the midline 30-40 mm behind the external occipital protuberance. The neck of the sheep was flexed and the needle advanced toward the tip of the animal's nose until cerebrospinal fluid was obtained. The cerebrospinal fluid often flowed from the needle under internal pressure, otherwise slight suction was necessary to withdraw the sample. For homovanillic acid analysis only clear 3-5 ml samples uncontaminated by blood were used.

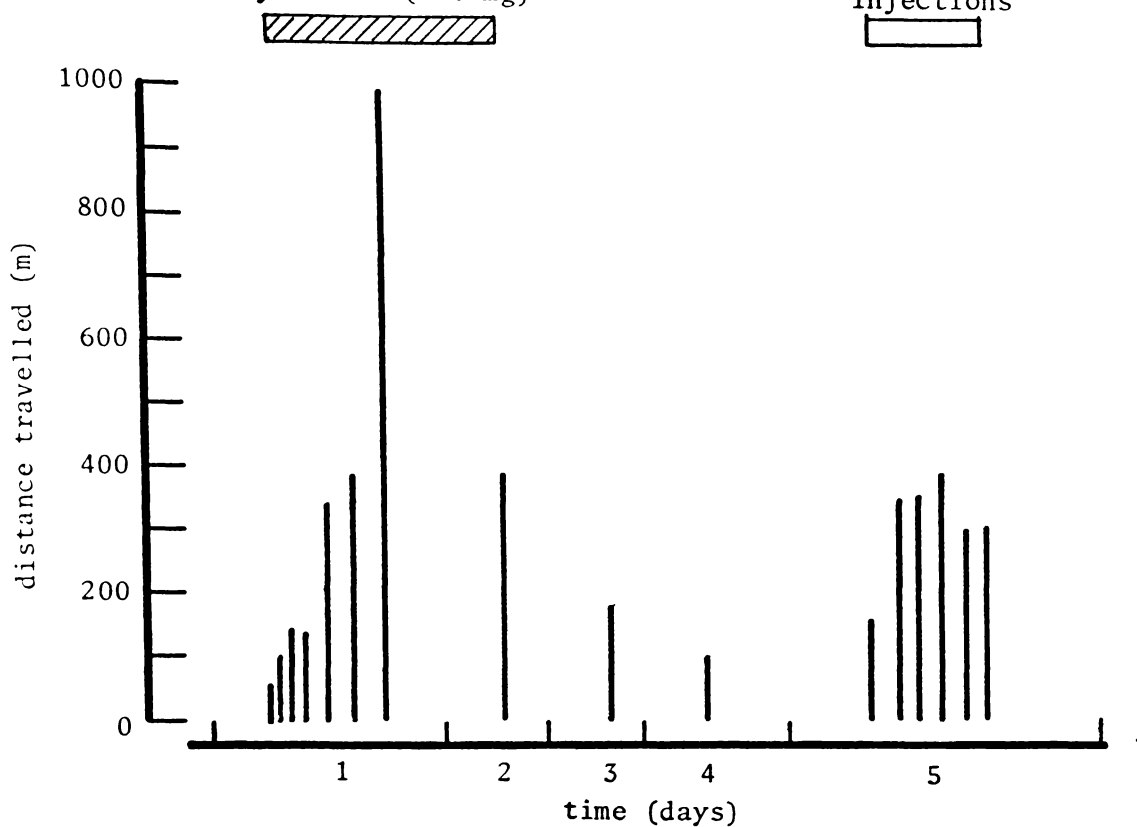
2 hourly 1-dopa
injections (100 mg)2 hourly saline
injections

Figure 8-1a. Distance covered by sheep A before the onset of staggers symptoms when chased by a dog at intervals over 5 days (with 1-dopa injections on day 1 and saline injections on day 5).

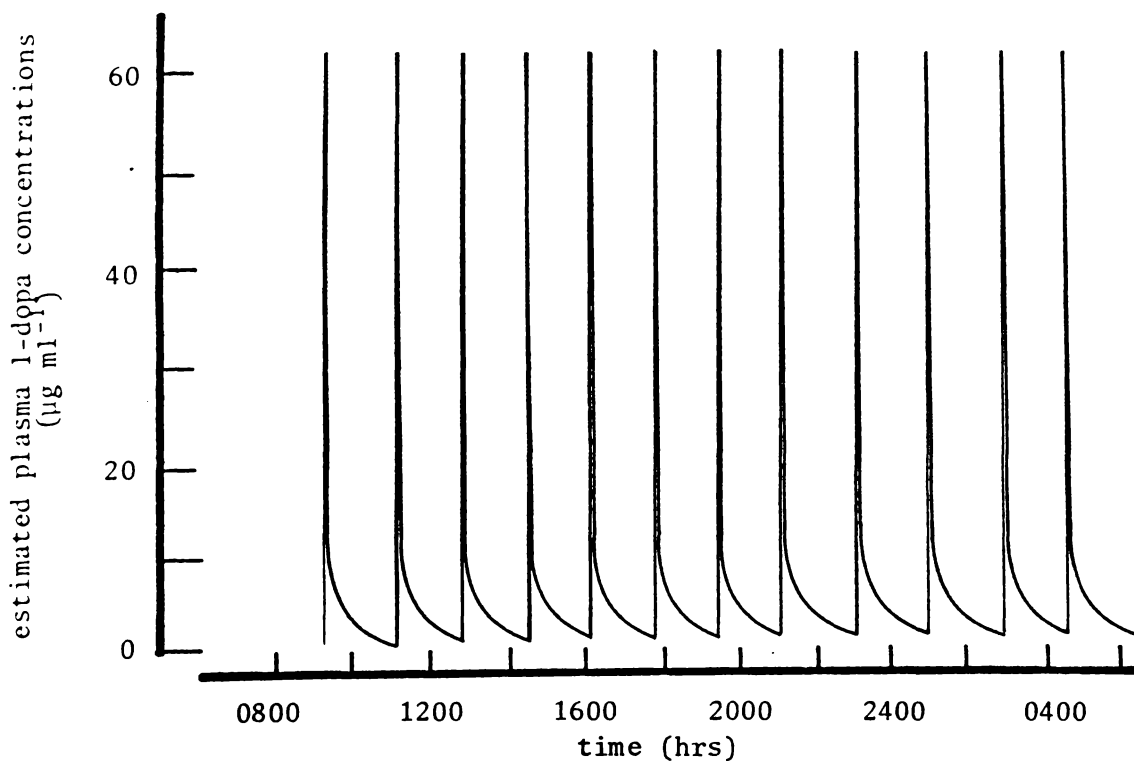


Figure 8-1b. Estimated plasma concentrations of 1-dopa after 100 mg injections of 1-dopa at 2 hourly intervals using pharmacokinetic model of §5.3.

The cerebrospinal fluid of some sheep with ryegrass staggers was viscous and yellow compared to the clear water-like samples found in all healthy sheep. The analyses gave the following results.

	Homovanillic Acid (ng ml ⁻¹)	Lactic Acid (mg dl ⁻¹)
Controls	32.4±16.0 (5)	31.7±5.7 (8)
Ryegrass staggers	6.9± 4.6 (5)	35.3±5.1 (10)
	(numbers analysed in parentheses)	

There was no significant difference between the lactic acid levels in the 2 groups ($p>0.5$, Kolmogorov-Smirnov 2-sample test), but the reduction in homovanillic acid in the staggers-affected sheep is significant ($p<0.01$, Kolmogorov-Smirnov 2-sample test).

DISCUSSION

The lack of any effect of adrenaline in increasing or of propranolol in reducing the severity of staggers symptoms argues against an essential peripheral catecholamine component in the stress-aggravated precipitation of ryegrass staggers. This conclusion is in contrast to the evidence relating to the effects of these agents in Parkinson's disease (Herring 1964, Owen & Marsden 1965).

The improvement in the staggers-affected sheep with l-dopa is interesting but the significance of this result is obscured by the lack of concurrent controls. The improvement seen cannot presently be attributed to the l-dopa as some adaptive mechanism to the repeated testing regime may also account for the observed changes (Figure 8-1a). The testing on day 5 after saline injections would suggest that at least some adaption may occur and the difference between the l-dopa and saline infusions could result either from differences between the tests (pressure of the dogs, environmental conditions) or to physiological differences in the tremorgen activity on the different days.

The question of the efficacy of intravenous l-dopa in the treatment of Parkinson's disease is also relevant to this experiment. Hornykiewicz (1973, 1974) is of the opinion that a single intravenous injection of l-dopa (in contrast to oral l-dopa) has immediate effects in reducing akinesia and rigidity in Parkinson's disease but there is some evidence to the contrary (Fehling 1966). To obtain relief with oral l-dopa on the other hand a long slow build up in the dose is necessary over weeks or months (Cotzias *et al.* 1969) in order to minimize the side effects that result from the large doses necessary by this route of administration (Klawans & Bergen 1975). In view of the complete absence of data on the ability of l-dopa to survive passage through the gut of ruminants to the small intestine (where absorption into the bloodstream is concentrated (Wade *et al.* 1973)) intravenous injection was the method of choice in the present experiment. An investigation is needed to examine the pharmacokinetics of oral l-dopa in sheep.

The minimum circulating level of l-dopa in the plasma for the effective control of Parkinsonian symptoms appears to be around $1 \mu\text{g ml}^{-1}$ (Tolosa *et al.* 1973, Sweet & McDowell 1974, Bianchine & Shaw 1976). To a first approximation the time course of the plasma l-dopa in the injected sheep may be predicted from the pharmacokinetic data of §5.3 given that the sheep were approximately the same weight and age as those from which the kinetic parameters were derived. The plasma concentration reached by a dose of 100 mg (X_0) in a central compartment volume of V_c is given by

$$C = \frac{X_0}{V_c} = \frac{100\ 000}{1627} = 61.5 \mu\text{g ml}^{-1}$$

The estimated time course of the l-dopa plasma concentration in the present experiment is plotted in Figure 8-1b. It may also be shown that a concentration of $1 \mu\text{g ml}^{-1}$ is reached after 118 minutes, which ensures an effective dose in terms of a central dopamine deficiency over the injection regime described (Figure 8-1b).

The fact that no difference was discernible between the lactic acid levels of the 2 groups indicates no gross changes in central carbohydrate metabolism, although the cerebrospinal fluid lactic levels are higher in these sheep than the normal values reported for humans of 19 (11-27) mg dl⁻¹ (Glaser 1926). The homovanillic acid levels in staggers-affected sheep closely correspond to levels in patients with Parkinson's disease (12±9 ng ml⁻¹) (Parkes *et al.* 1971). In normal human controls a value of 39±13 ng ml⁻¹ was found.

CONCLUSIONS

The results of these preliminary experiments are interesting but do not indicate one way or another whether the catecholamine hypothesis of ryegrass staggers is supportable. However the homovanillic acid levels indicate that a more thorough study of cerebrospinal and brain catecholamine metabolites is warranted. It has also become clear that progress in staggers research is impeded by the lack of an objective test for symptom severity. Some of the techniques used in the evaluation of Parkinsonism treatment (Calne 1970) could be useful in developing such tests. If we accept for the moment that fungal toxins are the cause of ryegrass staggers and also that they are acting through a dopaminergic mechanism, then their rapid (2-10 minutes) action when administered intravenously (Gallagher *et al.* 1977) makes dopamine vesicle depletion unlikely. Synthesis inhibition can be rejected for the same reason. Simple blockage of receptors would tend to raise brain homovanillic acid levels rather than lower them if the effects of dopaminergic blocking drugs can be extrapolated although these drugs may also impair the elimination of acid metabolites from the brain (Carlsson & Lindqvist 1963, Andén *et al.* 1964). This leaves interference with the neural release, by one means or another, as a possible mechanism.

8.3 HALOPERIDOL-INDUCED STEREOTYPED BEHAVIOUR IN SHEEP

INTRODUCTION

Neuroleptic drugs (dopaminergic receptor blockers) including the butyrophenone derivative haloperidol are effective inhibitors of stereotyped ingestive behaviour seen in rats and other animals after the injection of high doses of amphetamine or apomorphine (Randrup & Munkvad 1974, Randrup *et al.* 1975, Rollema *et al.* 1976) and indeed this property is used as a test of neuroleptic activity (Janssen 1965). Other forms of physiologically (Janssen 1965, Antelman *et al.* 1975, Zis & Fibiger 1975) or electrically (Phillips & Nikaido 1975) stimulated feeding behaviour are also depressed by neuroleptics.

Haloperidol is a central nervous system depressant used clinically as a tranquilizer in low doses (Janssen 1965). In higher doses it causes akinesia, catalepsy and tremor in a variety of mammals (Janssen 1965, Oberst & Crook 1967, Timsit 1967). Dose dependent impairment of conditioned avoidance response (Janssen 1965, Oberst & Crook 1967), ability to perform prolonged physical exercise (Oberst & Crook 1967), grooming activity (Rohte 1969) and certain operant behaviour (Monti & Hance 1967) have also been reported. Neuroleptics are thought to exert their effects principally through competitive blockade of postsynaptic dopaminergic receptors (Andén *et al.* 1970, Cools & van Rossum 1970, Cools 1971, Rollema *et al.* 1976). In high doses haloperidol also blocks noradrenergic receptors (Andén *et al.* 1970).

In view of this background stereotyped ingestive behaviour discovered in sheep after intravenous administration of a comparatively high dose of haloperidol ($1.5\text{--}2.0\text{ mg kg}^{-1}$) was unexpected and has not been reported elsewhere. This behaviour has been observed on 5 separate occasions involving a total of 30 sheep. Intravenous haloperidol at doses of 0.5 and 1.0 mg kg^{-1} causes motor inco-ordination and catalepsy but is insufficient to cause stereotyped behaviour.

METHODS

In this study 4 Dorset Horn sheep (50-60 kg) were injected with haloperidol (2 mg kg^{-1}) in a 1% lactic acid solution and 4 others with vehicle alone. Together with 7 non-injected controls the sheep were brought indoors from pasture and subjected to a series of tests, namely, response to humans, reactions to a barking dog at close quarters as individual sheep and in a group, an auditory startle test, and a food reaction test involving 5 different foodstuffs. The sheep were also tested in the field for their responses to driving before a dog.

RESULTS

The injection of vehicle alone caused no changes in behaviour. Within 5 minutes catalepsy and tranquilization developed in the sheep injected with haloperidol (Plate 8-1a). The second phase began at 8 to 10 minutes as jaw movements in the half-head height position and was rapidly followed by lowering of the head to ground level and intense licking and gnawing which continued almost without stop for up to 12 hours (Plate 8-1b). Chewing of available objects at the ground level such as the wool or hooves of other sheep (Plate 8-1c) or their own front legs was commonly seen when these objects were within the radius of head movement. Clockwise circling and walking backwards were also observed. Some sheep lay down but continued with this incessant ingestive response pattern. They laid prone unless lifted to their feet where they could remain in an upright position without additional support. On pasture the sheep moved very little and could not be driven. Grass was ingested but normal discrimination was absent, rather a circle of grass was chewed out to ground level. Grass pulled out by the roots was ingested while the roots and attached earth were rejected from the mouth.

When 5 foods were laid out at 70 cm intervals, the control sheep showed a discrimination, eating in order of preference, crushed barley, a standard dairy meal, hay and cut grass and avoiding meat meal. The haloperidol-treated sheep showed no awareness



a) Cataleptic phase of short duration 5 minutes after haloperidol injection.



Licking and gnawing behaviour on a concrete floor.



Biting the hoof of another sheep.

ate 8-1. Stereotyped ingestive behaviour of Dorset Horn sheep on a concrete floor after 2 mg kg^{-1} (iv) of haloperidol.

of the presence of food but ate without preference when placed with their heads over the food. A slight discrimination against meat meal seemed to be present but it was still ingested by 3 of the 4 treated sheep.

The haloperidol-treated sheep were completely unresponsive to the presence of other sheep, humans or a dog. Over the first 6 hours postinjection they did not move away from a dog nor did they show any reaction to dog barking or noise during an auditory startle test. After 6-8 hours, awareness of the presence of the dog began to occur but flocking behaviour, reactions to human presence and a normal startle response to a loud noise did not return to a significant degree for 10-12 hours. All overt behavioural effects of the drug had disappeared after 24 hours. However the 4 treated animals showed a reduced flight distance to a human handler feeding the sheep supplement at pasture and would stand to be stroked by hand, a highly atypical response compared with the other sheep. This effect persisted for over 2 months with little sign of extinction.

DISCUSSION

Intensive eating in a variety of wild African ungulates in response to the neuroleptics azaperone and fluanisone (Pienaar 1968) suggests that this behaviour may not be confined to sheep alone nor specifically to haloperidol. Meldrum *et al.* (1977) have reported transient dystonia and vigorous gnawing and licking in a small minority of baboons treated with haloperidol. These responses were abolished by intravenous administration of anticholinergic drugs. While little information is available on the effects of central nervous stimulants on the behaviour of domestic animals, the direct dopamine receptor agonist apomorphine produces stereotyped ingestive behaviour in cattle and sheep (Sharman & Stephens 1974) similar to that seen with haloperidol. However apomorphine-treated sheep were reported to concentrate their chewing and licking above ground level.

The considerable body of behavioural and neurochemical evidence relating to amphetamine and apomorphine-induced stereotypy suggests that the stimulation of central catecholamine neurones and in particular, the basal ganglia dopaminergic tracts, is essential to the expression of stereotyped behaviour (Ernst & Smelik 1966, Randrup & Munkvad 1974, Randrup *et al.* 1975). But clearly the induction of similar behaviour by a dopamine receptor antagonist described in this report has a different neurochemical explanation. With dopaminergic blockage, nor-adrenergic pathways considered by some to be involved in both feeding (Ritter & Epstein 1975) and stimulant stereotypy (Costall & Naylor 1972) could be freed from inhibition. However disinhibition appears unlikely if, at the dose level of haloperidol used in this study, noradrenaline receptors were also blocked. To explain this apparent contradiction Meldrum *et al.* (1977) have advanced the hypothesis that the transient dystonia and ingestive behaviour sometimes seen in man and baboons after neuroleptics are due to an over-stimulation of a subset of striatal dopamine receptors which are inadequately blocked by the drug.

"Displacement activities" described by Kortlandt (1940) and Tinbergen (1952) are components of the behavioural repertoire of birds and animals and are relatively stereotyped in their expression. Mildly induced stress from open-field testing of individual sheep frequently leads to a period of "sham" or "displacement" grazing (Kilgour 1969) and similar behaviour has been reported between bouts of fighting in dairy cows (Schein & Fohrman 1955) and deer (Müller-Using 1952). Such displacement activities have been explained by a disinhibition theory (Fentress 1973) where 2 drives or major classes of behaviour each independently inhibiting a third lose their inhibitory capacity when simultaneously activated and allow the third to be freely discharged. Although grazing will be a strongly encoded behaviour in a ruminant such as the sheep other reactions related to survival which depend on responsiveness to such environment stimuli as the normal reactions to

dogs (predators) will take precedence over grazing. It could be argued that haloperidol in suppressing these behaviours allows the persistent expression of a specific behavioural complex analogous to the intermittent bouts of "displacement grazing".

8.4 TAMING EFFECT OF HALOPERIDOL

INTRODUCTION

The most unexpected outcome of the previous experiment was the independent observation by a stockman of a residual and persistent taming effect of haloperidol expressed as a reduction in flight distance to zero. Flight distance in this context is defined as the minimum distance that the sheep will permit human approach before retreating. The possible existence of such a phenomenon is of considerable interest especially in view of a current programme to develop taming and training techniques for sheep to act as leaders or "Judas" sheep in abattoirs.

The use of a neuroleptic to accelerate social conditioning among conspecifics is not new since the neuroleptic azaperone is used to prevent aggression when mixing strange pigs (Brutzel & Krienle 1970, Symoens 1970) and the practice has come into widespread use in Europe (Callear & van Gestel 1971). Doses of azaperone between 1.5 and 3 mg kg⁻¹ in pigs cause sedation lasting for about 2 hours after which time the mixing of pigs from different sources occurs with comparatively little fighting (Symoens 1970). In explanation it has been suggested that by the time the sedative effects of the drug have dissipated, the pigs become accustomed to one another by the exchange of sensory information, for instance smell, and hence accept the presence of strange pigs (Symoens 1970). This concept was stated to be in accordance with the blocking of central aggressive motor nervous transmission without impeding the penetration of specific sensory impulses to the cortex. There is however no direct evidence to support this contention. Another mechanism that

may contribute, and does not appear to have been considered is the very powerful endocrine change induced by neuroleptics. In general, and species differences do occur, gonadotrophin production is depressed (Dickerman *et al.* 1974), prolactin greatly increased (Dickerman *et al.* 1974), corticotrophin and corticosteroid increased (Balestreri *et al.* 1974b) while oxytocin release is variously reported as unaltered (Seybold & Miller 1977) or depressed (Clarke & Lincoln 1975). The physiological and behavioural effects of these hormones such as the modification of conditioned responses by corticotrophin (Endröczy 1972) or maternal bond formation by prolactin (Zarrow *et al.* 1971, Korányi *et al.* 1977) could play a role in the azaperone assimilation of strange pigs.

The idea that interspecies reactions may also be influenced by a neuroleptic is a novel concept and an experiment employing thirty 3-year old hill country wethers of mixed breed with little previous handling history was designed to test the hypothesis.

METHODS

The wethers were divided into 4 groups (3 groups containing 8 sheep each with 6 sheep in the remaining group) which were subsequently grazed, tamed and scored separately. Two technicians (trainers) undertook the taming of these sheep by the best means they could devise as long as equal daily attention was given to each sheep. The sheep were tamed by a familiarization process involving consistent daily contact and which was reinforced with crushed barley, proceeding progressively from a race to a pen and then to the open field. They were taught using simple operant techniques to touch the trainer's hand with their noses in return for back-scratching. Four hours per day over a 35 day period were devoted to the taming process. A "tameness" score was subjectively assigned daily to each sheep ranging from 1 representing an unapproachable sheep in a confined space such as a race to 9 when the animal was tame enough to

permit physical contact in the open field or even to approach the trainer itself. A second measure of tameness was attempted, an assessment of flight distance, but this was subject to uncontrolled environmental effects such as weather and surrounding farming operations and proved unreliable. After 10 days of handling to permit the assessment of tameness ratings for each sheep haloperidol was administered (2 mg kg^{-1}) intravenously as previously described (§8.3) and the treated sheep subjected to intensive handling and physical contact by 4 handlers over 8 hours. The 2 trainers were not present on the day of drug administration nor did they know the identity or the number of the treated sheep. Haloperidol was given to 18 sheep and saline to the remaining 12 distributed amongst the 4 taming groups as follows.

- group 1: All treated with haloperidol and handled (n=8).
- group 2: Half (n=4) treated with haloperidol and half (n=4) treated with saline. The haloperidol treated sheep were handled as in group 1. Since sheep learn quickly by example it was felt that should the drug treatment have a marked taming effect it would be of interest, in the weeks subsequent to treatment, to examine the facilitation effect of 4 treated sheep on the 4 saline treated sheep. In fact, since the drug treatment had only marginal effects no useful data could be gathered on this point.
- group 3: All treated with saline (n=8).
- group 4: All treated with haloperidol then isolated out of sight and sound of other sheep or humans for the duration of the 8 hours handling (n=6).

The injected sheep were mixed into a single flock for handling and were returned to their 4 original groups the following morning by the trainers. The saline treated sheep could not be handled in the way that the sedated sheep were handled but all sheep

(except for the 6 isolated sheep) were kept in close proximity to the 4 human handlers. For the purpose of comparing drug effects on tameness immediately before and after haloperidol treatment there are 3 separate treatment groups.

- (A) haloperidol treated and handled (n=12)
- (B) saline treated and non-handled (n=12)
- (C) haloperidol treated and non-handled (n= 6)

RESULTS AND DISCUSSION

The mean group tameness scores showed a fluctuating but overall progressive increase throughout the experiment (Figure 8-2). By comparison any effect of the haloperidol treatment (day 11) was minor. A one way analysis of the variance of the tameness scores between the 4 taming groups shows no significant differences ($p>0.2$) on days 1, 10, 12 or 35. However a close examination of the data from immediately before and after haloperidol treatment reveal a treatment influence (Tables 8-1 and 8-2) and, furthermore, the trainers correctly identified the treated groups on the basis of changes in the subjective tameness score ratings the day following treatment.

Table 8-1

Changes in Tameness Ratings After Haloperidol Treatment*

		No. of Sheep Whose Score			Mean** Score Change
		Increased	Same	Decreased	
group A	haloperidol (handled)	10	1	1	+0.90
group B	saline	6	2	4	+0.17
group C	haloperidol (isolated)	3	1	2	-0.08

Overall change in mean tameness score = +0.41

* between tameness assessments on days 10 and 12

** [F = 4.11, $0.01 < p < 0.05$, analysis of variance between groups]

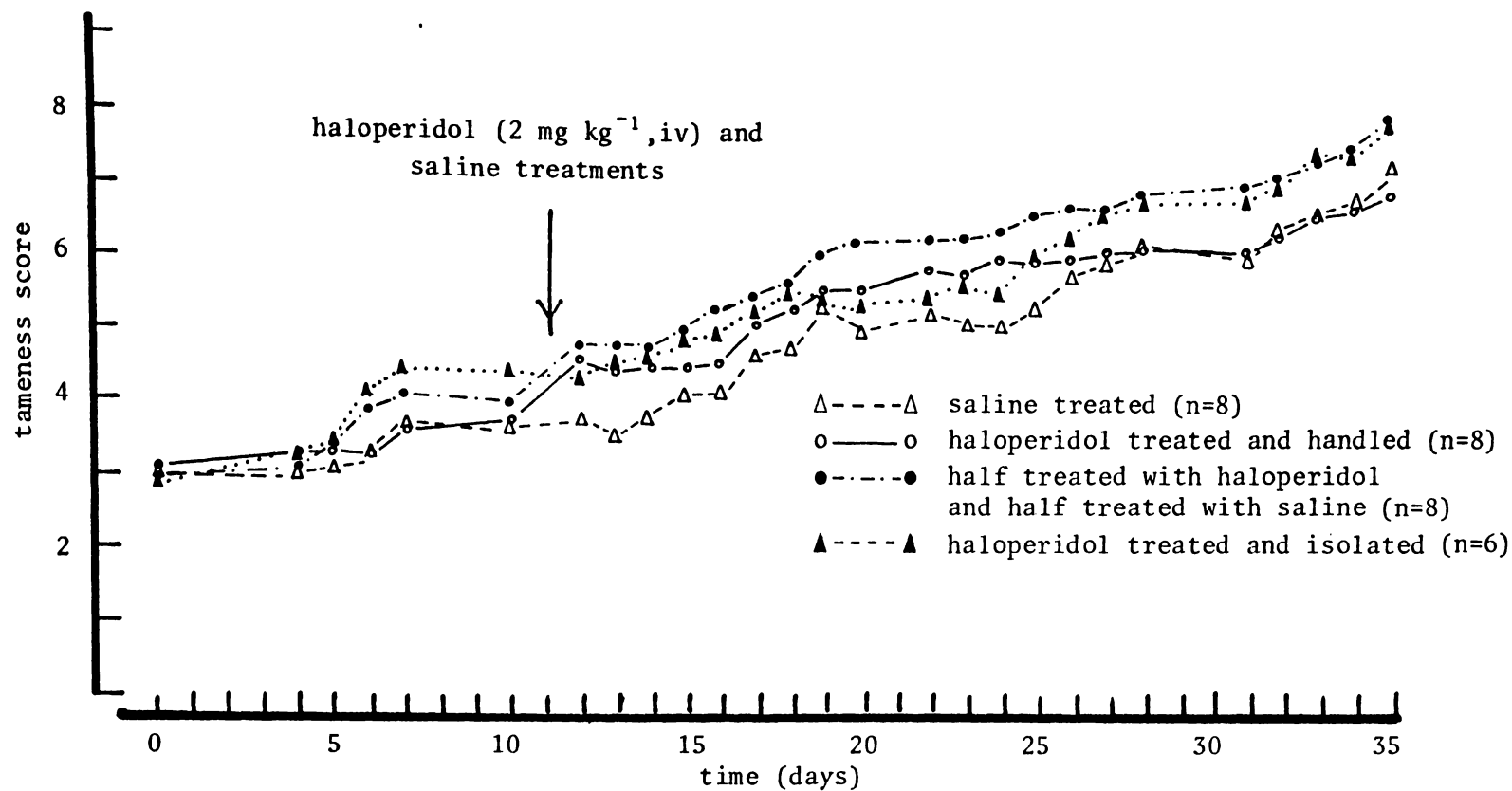


Figure 8-2. The effects of daily handling and haloperidol treatment (day 11) on sheep tameness score.

Table 8-2

Distribution of Changes in Tameless Ratings in the 12 Haloperidol-treated and Handled Sheep (Group A) Before and After Treatment

		No. of Sheep Whose Score			Mean
		Increased	Same	Decreased	Score Change
Preceding Test	(day 10)	2	10	0	+0.17
After Treatment	(day 12)	10	1	1	+0.90
Subsequent Test	(day 13)	2	6	4	+0.04

An analysis of variance of the changes in tameness scores before and after treatment between the 3 treatment groups (A versus B versus C) indicates a significant difference ($F_{2,27} = 4.11$; $0.01 < p < 0.05$). The difference between the changes in mean tameness scores of the 2 non-handled groups (B minus C) (0.25 ± 0.24 SE) is non-significant ($p > 0.2$, 2-tailed t test). On the other hand comparing the treated and handled (group A) versus the non-handled groups (groups B and C) the difference is 0.85 ± 0.17 (SE) and is highly significant ($p < 0.001$).

These results suggest a treatment effect is present; that drug treatment alone without handling does not produce an effect (and also that the change in tameness scores cannot be attributed to a residual sedation resulting from the haloperidol). As expected the handling of the sheep while under the influence of haloperidol is an essential component of the accelerated conditioning.

One sheep (LY2375) showed a very marked increase in tameness (of the same order as observed in the previous experiment (§8.3) from being only cautiously approachable to being tame (zero flight distance) in the pen. The magnitude of this increase was unmatched by any other animal over any 2 day period in the course of the experiment.

Overall however the neuroleptic effect was not as evident as previously observed (§8.3) for reasons that are not clear. Certainly the drug treatment has had no effect on the tameness rating of the sheep at the end of the experiment (Figure 8-2). Ewes may be more susceptible than wethers or it may be that the response of sheep already accustomed to a degree of handling is greater. It may be significant in this respect that the sheep exhibiting the greatest increase in social conditioning already had the highest rating of any sheep before haloperidol administration. The dose level in retrospect may also have been suboptimal in view of the reported dose dependent responses of pigs during mixing (Symoens 1970).

8.5 CONCLUSIONS

More questions have been raised than answered in this chapter though certain points can be made. The ubiquitous nature of catecholamine involvement in central nervous system physiology has been demonstrated by the locomotive, ingestive and social conditioning responses seen in sheep after the administration of a dopaminergic receptor blocker. A thorough examination of the changes in central neurochemistry in sheep affected by ryegrass staggers in the light of the reduced homovanillic acid level found in these animals is indicated in conjunction with a study of the central effects of the suspected fungal tremorgens. The behaviour of sheep under the influence of haloperidol raises important theoretical questions about the present understanding of stereotyped behaviour and the central mechanism of action of neuroleptic drugs in general. The results of the social conditioning experiment were not clearcut but the original observation suggests that under certain still poorly defined conditions a real and long term taming effect may exist. Further work in this field is indicated perhaps in combination with a study of the endocrine effects of neuroleptics in sheep.

CONCLUSIONS

The history of catecholamine research may be viewed as evolving through a succession of stages of increasing insight from Cannon and Selye to the recognition that we must take into consideration a broadening array of multi-interdependent variables from both a behavioural and endocrinological viewpoint if we are to understand the nexus between emotional behaviour and physiological changes and ultimately the productive output of domestic stock.

This thesis has been concerned with the relationships of the catecholamines to physiological conditions in sheep in a variety of situations. The present state of knowledge of the catecholamines has been reviewed and applied to a number of specific areas relating to sheep. The 2-fold importance of the catecholamines in the study of sheep physiology is emphasized throughout; their role as dependent variables reflecting the influence of the environment and as independent variables affecting the physiological state of the organism.

The conclusions arising from each experiment are recorded together with the results after each major section of the thesis but the new findings can be summarized as follows:

Catecholamine Infusions Hitherto unreported interrelationships between blood pressure, catecholamines and corticosteroids have been found during and after catecholamine infusions. The physiological connections between the catecholamines, energy substrates and other hormones are emphasized and the pharmacokinetics of 1-dopa in sheep plasma after its intravenous administration described.

Cannulation and Recovery The time necessary for sheep adapted to an indoor environment to recover from the surgical implantation of carotid and jugular cannulae has been found to be 48 hours.

Transport Experiment A complete and detailed description of the plasma profiles of several blood substances in stock travelling between the farm and the abattoir has been achieved for the first time. These results are discussed in terms of the physiological and endocrinological background of the measured substances. Experiments of this nature make possible the objective formulation of regulations for humane and efficient stock handling.

Electrical Stunning The essential role of the catecholamines in the metabolic and physiological consequences of electrical stunning has been demonstrated by the high levels of catecholamine release into the bloodstream post-stunning and the effectiveness of adrenergic blocking drugs in the prevention of blood splash. Abattoir handling does not account for the observed differences in blood splash incidence between mobs of lambs but other factors in their previous history may account for part of the difference. Some interesting endocrinological events associated with the double electrical stunning of cannulated lambs at 2 hourly intervals have also been demonstrated.

Central Nervous System The behavioural effects of haloperidol in sheep, discovered in this investigation, call into question the exact mode of action of this drug and also previously proffered explanations of the basis of stereotyped behaviour. The possible taming effects of haloperidol and the role of the catecholamines in ryegrass staggers need further detailed study.

Assay of Catecholamines Improvements to the precision of the catecholamine assay and in the speed of data analyses have resulted from new extraction and assay techniques and a computer programme for data processing developed during this study.

The catecholamines have an important place in the study of behavioural arousal since their release is less subject to the intermediate past history of the animal than are the corticosteroids. Their pulsatile release however makes collection at the appropriate times

difficult. New methods of sample collection must be devised. Developments in electronics and the reduction in the necessary blood sample volume mean that the concept of collecting blood samples by remote control from freely moving animals is a practical possibility, given the development of the necessary mechanical system and the problems of preventing blood clotting and catecholamine catabolism in whole blood can be solved. Such an approach is essential in the long term in view of the need to collect large numbers of samples at specific times and to minimize sampling stressors. During the currency of this work development of the radioenzymatic assay of catecholamines has reached the point where the previous problem of sensitivity has been overcome. Future studies can thus be undertaken to examine situations in which very small changes in concentration of the catecholamines occur. Some areas relating to problems in agricultural research such as stress assessment and meat processing have been explored but there exist a number of other problems under active scientific investigation to which the knowledge of central and peripheral catecholamines should be applied.

Reproduction The gonadotrophic and pineal hormones involved in the physiological and behavioural control of the reproductive cycle are under active catecholaminergic control. Many drugs active in catecholamine biochemistry alter or disrupt reproductive function.

Lactation Pituitary prolactin secretion is partly regulated by a prolactin inhibiting hormone and some evidence suggests that this may be dopamine. Thus increasing dopamine release inhibits prolactin while decreasing dopamine release or blocking dopamine receptors leads to elevated prolactin levels and can induce lactation. The questions surrounding the adrenergic inhibition of the milk ejection reflex have not been adequately investigated.

Metabolic Diseases Metabolic diseases including pregnancy toxæmia and grass tetany are often precipitated by environmental stressors. In relation to the hypocalcaemia of grass tetany for example it is known that adrenergic activation reduces blood calcium levels but the mechanism of action of this effect is not understood in a practical context.

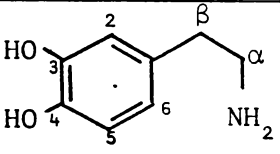
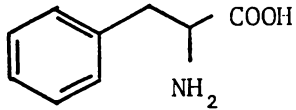
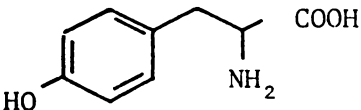
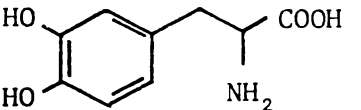
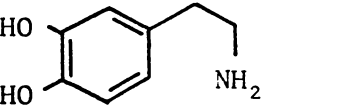
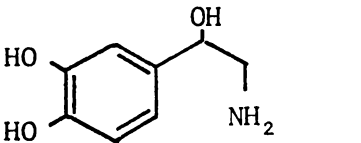
Neurological Diseases There is a need to develop a range of assays for the various catecholamine metabolites found in the central nervous system (and for other neurotransmitter systems) in order to investigate the aetiology of ryegrass staggers and other neurological diseases affecting domestic stock.

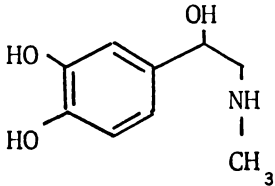
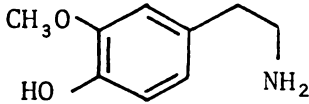
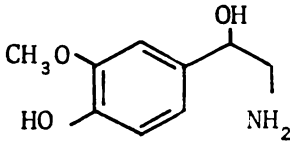
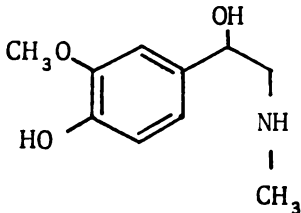
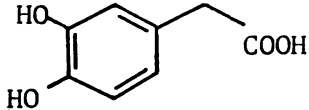
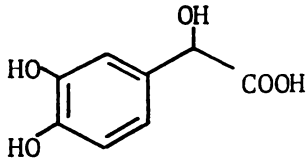
Behaviour and Development The effects of catecholamines on animal behaviour, development and learning and the complex interactions between changes in catecholamine turnover, caused by environmental stimuli, and productivity are not well understood. Many data can however be extrapolated from human and small animal studies.

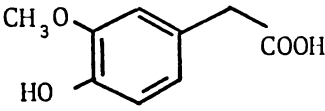
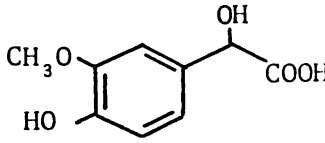
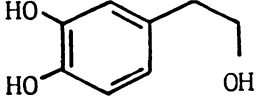
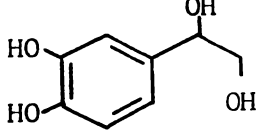
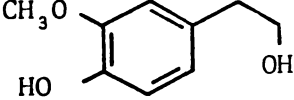
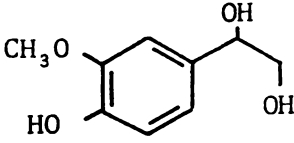
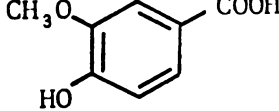
Meat Quality Links between ante-mortem handling, catecholamine release and postmortem meat quality have been established but the practical significance of preslaughter handling and killing procedures in the New Zealand situation has not been adequately assessed.

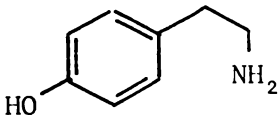
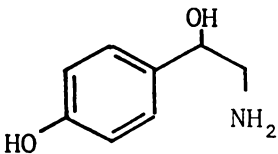
The present studies with the sheep have demonstrated a similar catecholamine physiology to that found in other studied species. By the same token the results presented in this thesis may well provide some new insights for physiologists working with other animals.

STRUCTURE AND ABBREVIATIONS OF THE
CATECHOLAMINES, THEIR PRECURSORS AND METABOLITES

Compound	Abbreviation	Structure and Formula	Molecular Weight
			
phenylalanine	PHE	 $C_9H_{11}NO_2$	165.19
tyrosine	TYR	 $C_9H_{11}NO_3$	181.19
3,4-dihydroxyphenyl- alanine	L-DOPA	 $C_9H_{11}NO_4$	197.19
dopamine (3,4-dihydroxyphenyl ethylamine)	DA (DM)	 $C_8H_{11}NO_2$	153.18
hydrochloride		$C_8H_{11}NO_2 \cdot HCl$	189.65
hydrobromide		$C_8H_{11}NO_2 \cdot HBr$	234.10
picrate		$C_8H_{11}NO_2 \cdot C_6H_3N_3O_7$	382.29
noradrenaline (norepinephrine)	NA (NE)	 $C_8H_{11}NO_3$	169.18
hydrochloride		$C_8H_{11}NO_3 \cdot HCl$	205.65
bitartrate monohydrate		$C_8H_{11}NO_3 \cdot C_4H_6O_6 \cdot H_2O$	337.29

Compound	Abbreviation	Structure and Formula	Molecular Weight
adrenaline (epinephrine)	A (E)	 $C_9H_{13}NO_3$ $C_9H_{13}NO_3 \cdot HCl$ $C_9H_{13}NO_3 \cdot C_4H_6O_6$	183.20 219.68 333.29
3-methoxytyramine	MT	 $C_9H_{13}NO_2$	167.21
normetanephrine	NMN	 $C_9H_{13}NO_3$ $C_9H_{13}NO_3 \cdot HCl$	183.20 219.68
metanephrine	MN	 $C_{10}H_{15}NO_3$ $C_{10}H_{15}NO_3 \cdot HCl$	197.23 233.70
3,4-dihydroxyphenyl- acetic acid	DOPAC	 $C_8H_8O_4$	168.15
3,4-dihydroxy- mandelic acid	DOMA	 $C_8H_8O_5$	184.15

Compound	Abbreviation	Structure and Formula	Molecular Weight
3-methoxy-4-hydroxyphenylacetic acid (homovanillic acid)	HVA	 $C_9H_{10}O_4$	182.18
3-methoxy-4-hydroxymandelic acid (vanillylmandelic acid, vanilmandelic acid)	VMA	 $C_9H_{10}O_5$	198.18
3,4-dihydroxyphenyl-ethanol	DHPE (DOPET)	 $C_8H_{10}O_3$	154.17
3,4-dihydroxyphenyl-glycol	DHPG (DOPEG)	 $C_8H_{10}O_4$	170.17
3-methoxy-4-hydroxy-phenylethanol	MHPE (MOPET)	 $C_9H_{12}O_3$	168.20
3-methoxy-4-hydroxy-phenylglycol	MHPG (MOPEG)	 $C_9H_{12}O_4$	184.20
3-methoxy-4-hydroxybenzoic acid (vanillic acid)	VA	 $C_8H_8O_4$	168.14

Compound	Abbreviation	Structure and Formula	Molecular Weight
tyramine			
		$C_8H_{11}NO$	137.18
hydrochloride		$C_8H_{11}NO \cdot HCl$	173.74
octopamine			
		$C_8H_{11}NO_2$	153.18
hydrochloride		$C_8H_{11}NO_2 \cdot HCl$	189.65

APPENDIX II

SOURCES, FORMULAS AND MOLECULAR WEIGHTS OF THE
CHEMICALS, BIOCHEMICALS AND DRUGS USED IN
THE EXPERIMENTAL INVESTIGATIONS

acetic acid (glacial) (AR) CH_3COOH MW 60.05
BDH Chemicals Ltd, Poole, UK

S-adenosyl-L-[methyl- ^3H] methionine MW 399 11.4 Ci mmole $^{-1}$
1 mCi ml $^{-1}$
Radiochemical Centre, Amersham, UK

L-adrenaline $\text{C}_9\text{H}_{13}\text{NO}_3$ MW 183.20
Sigma Chemical Company, St Louis, Missouri, USA

DL-[7- ^3H] adrenaline hydrochloride 10.8 Ci mmole $^{-1}$ 1 mCi ml $^{-1}$
Radiochemical Centre, Amersham, UK

alkylphenoxy polyethoxy ethanol (Triton X-100) (MG)
Rohn & Haas, Philadelphia, Pennsylvania, USA

aluminium oxide (CG) Al_2O_3 100-200 mesh MW 101.96
Hopkins & Williams Ltd, Chadwell Heath, UK

2-amino-2-(hydroxymethyl)-1,3-propanediol (AR) MW 121.14
Fluka AG, Buchs, Switzerland

ammonia (AR) (~25%) sp gr = 0.91 MW 17.03
BDH Chemicals Ltd, Poole, UK

antifoam B
Dow Corning, Auckland, New Zealand

L-ascorbic acid $\text{C}_6\text{H}_8\text{O}_6$ MW 176.13
E. Merck, Darmstadt, West Germany

catechol-O-methyltransferase (chromatographically purified
from pig liver) 1850 units per mg of solid and 2250
units per mg of protein
Sigma Chemical Company, St Louis, Missouri, USA

citric acid (AR) $\text{C}_3\text{H}_4(\text{OH})(\text{COOH})_3 \cdot \text{H}_2\text{O}$ MW 210.14
May & Baker, Dagenham, UK

cupric sulphate (AR) $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ MW 249.68
BDH Chemicals Ltd, Poole, UK

L-cysteine hydrochloride (AR) $\text{CH}_2(\text{SH}) \cdot \text{CH}(\text{COOH}) \cdot \text{NH}_2 \cdot \text{HCl}$ MW 157.62
BDH Chemicals Ltd, Poole, UK

- diethyl ether (AR) $(C_2H_5)_2O$ MW 74.1
J.T. Baker Chemical Company, Phillipsburg, New Jersey, USA
- dimethylformamide (LR) $H.CO.N(CH_3)_2$ MW 73.10
Ajax Chemicals Ltd, Sydney, Australia
- 2,5-diphenyloxazole (PPO) (SG) MW 221.25
Packard Instrument Co. Inc, Downers Grove, Illinois, USA
- disodium ethylenediamine tetraacetic acid (AR)
 $C_{10}H_{14}N_2Na_2O_8.2H_2O$ MW 372.24
BDH Chemicals Ltd, Poole, UK
- disodium hydrogen phosphate Na_2HPO_4 MW 141.96
E. Merck, Darmstadt, West Germany
- L-dopa $C_9H_{11}NO_4$ MW 197.19
Sigma Chemical Company, St Louis, Missouri, USA
- L-[3H] dopa (L-3,4-dihydroxyphenyl [2,3- 3H] alanine
 2.5 Ci mmole^{-1} 1.0 mCi ml^{-1}
Radiochemical Centre, Amersham, UK
- dopamine hydrochloride $C_8H_{11}NO_3.HCl$ MW 189.65
Sigma Chemical Company, St Louis, Missouri, USA
- [3H] dopamine hydrochloride (3,4-dihydroxy [ring-G- 3H]
phenylethylamine hydrochloride) 5.0 Ci mmole^{-1} 1.0 mCi ml^{-1}
Radiochemical Centre, Amersham, UK
- ethyl acetate (LR) $CH_3.COO\ C_2H_5$ MW 88.11
BDH Chemicals Ltd, Poole, UK
- haloperidol $C_{21}H_{23}ClFNO_2$ MW 375.88
Janssen Pharmaceutica, Beerse, Belgium
- homovanillic acid $C_9H_{10}O_4$ MW 182.0
Sigma Chemical Company, St Louis, Missouri, USA
- hydrochloric acid (AR) (35.4%) sp.gr. 1.18 MW 36.46
BDH Chemicals Ltd, Poole, UK
- 4-hydroxydiphenyl (p-phenylphenol) (LR) $C_6H_5.C_6H_4.OH$ MW = 170.21
Hopkins & Williams, Chadwell Heath, UK
- iodine (resublimed) (LR) MW 126.90
BDH Chemicals Ltd, Poole, UK
- DL-lactic acid (AR) $CH_3.CHOH.COOH$ MW 90.08
BDH Chemicals Ltd, Poole, UK
- lithium lactate (LR) $CH_3.CHOH.COOLi$ MW 96.01
BDH Chemicals Ltd, Poole, UK

- DL-metanephrine hydrochloride $C_{10}H_{15}NO_3.HCl$ MW 233.70
Sigma Chemical Company, St Louis, Missouri, USA
- 3-methoxytyramine hydrochloride $C_9H_{13}NO_2.HCl$ MW 203.6
Sigma Chemical Company, St Louis, Missouri, USA
- L-noradrenaline bitartrate monohydrate $C_8H_{11}NO_3.C_4H_6O_6.H_2O$
MW 337.29
Sigma Chemical Company, St Louis, Missouri, USA
- DL-[7- 3H] noradrenaline hydrochloride 10.7 Ci mmole $^{-1}$ 1.0 mCi ml $^{-1}$
Biochemical Centre, Amersham, UK
- DL-normetanephrine hydrochloride $C_9H_{13}NO_3.HCl$ MW 219.68
Sigma Chemical Company, St Louis, Missouri, USA
- orthophosphoric acid (AR) H_3PO_4 sp gr 1.75 MW 98.00
BDH Chemicals Ltd, Poole, UK
- perchloric acid (AR) sp gr 1.70 $HC10_4$ MW 100.46
BDH Chemicals Ltd, Poole, UK
- 1,4-bis-2-(5-phenoxazolyl)-benzene (POPOP) (SG) MW 364.39
Packard Instrument Co. Inc, Downers Grove, Illinois, USA
- phentolamine mesylate (Rogitine) $C_{17}H_{19}N_3O.CH_3SO_3H$ MW 377.50
10 mg ml $^{-1}$
Ciba Laboratories, Horsham, UK
- potassium dichromate (LR) $K_2Cr_2O_7$ MW 294.19
BDH Chemicals Ltd, Poole, UK
- potassium ferricyanide (LR) $K_3Fe(CN)_6$ MW 329.26
BDH Chemicals Ltd, Poole, UK
- propranolol hydrochloride (Inderal) $C_{16}H_{21}NO_2.HCl$ MW 295.80
ICI Pharmaceuticals, Macclesfield, UK
- sodium chloride (AR) $NaCl$ MW 58.44
BDH Chemicals Ltd, Poole, UK
- sodium dihydrogen phosphate (AR) $NaH_2PO_4.2H_2O$ MW 156.01
May & Baker Ltd, Dagenham, UK
- sodium heparin 5000 units ml $^{-1}$
Commonwealth Serum Laboratories, Melbourne, Australia
- sodium hydroxide (AR) $NaOH$ MW 40.00
Ajax Chemicals, Sydney, Australia

sodium phenobarbitone (Nembutal) 60 mg ml⁻¹ C₁₂H₁₁N₂NaO₃
MW 254.20

Abbott Laboratories, Chicago, Illinois, USA

sodium sulphite (AR) Na₂SO₃ MW 126.04

J.T. Baker Chemical Co, Phillipsburg, New Jersey, USA

sulphuric acid (conc) (AR) H₂SO₄ sp gr 1.84 MW 98.08

BDH Chemicals Ltd, Poole, UK

toluene (AR) C₆H₅.CH₃ MW 92.14

BDH Chemicals Ltd, Poole, U.

trichloroacetic acid (AR) CCl₃COOH MW 163.39

BDH Chemicals Ltd, Poole, UK

zinc sulphate (AR) ZnSO₄.7H₂O MW 287.54

Peking Chemical Works, Peking, China

AR	analytical reagent
CG	chromatography grade
LR	laboratory reagent
MG	manufacturing grade
SG	scintillation grade
sp gr	specific gravity
MW	molecular weight
Ci	Curie

APPENDIX III

DATA ANALYSIS FOR THE FLUORIMETRIC CATECHOLAMINE ASSAY

A specific purpose FORTRAN IV computer programme (RXPC) was developed to integrate and analyze the recovery and fluorimetric data from the plasma catecholamine samples assayed as described in §4.4.

Table III-1

Programme RXPC Specifications

Computer	ICL 2903
Language	FORTTRAN IV
Lines	487
Peripherals	Card Reader, Line Printer

RXPC was constructed in such a manner as to meet certain objectives.

1. To impose minimal restraints on the analyst regarding the number, combination and types of catecholamines analyzed in any assay series.
2. To make the input of data as simple and as unregimented as possible.
3. To incorporate error checking in such a way as to flag errors where these occur (either in the data input or in its subsequent manipulation) but to continue processing of the remaining samples. Thus where errors occur they may all be corrected on the subsequent processing.
4. To present the output in a tabular and graphical form for easy and rapid comprehension.

5. To incorporate the facility to allow for missing data (eg. missing duplicates due to problems in the collection or assay of a sample or to error conditions arising in data processing).

Data Input

The input deck of cards consists of 7 card types, 6 of which are identified by the character in column 1. There are no constraints on the ordering of these cards except that the assay parameters must be placed before the assay data. The following characters are used to identify the various input data.

"C" is used to identify a comment card describing the experiment or containing any information required to be output via the lineprinter. There is no limit to the number of the comment cards.

Format

C ANY ALPHANUMERIC COMMENT ...

	↑↑	↑
Card column →	12	80

"D" indicates the end of a particular subset of samples. On encountering this card, mean values and standard deviations for the entered samples are calculated and the results tabulated and graphed before commencing with any subsequent subset. A maximum of 120 samples may be processed within any sample subset.

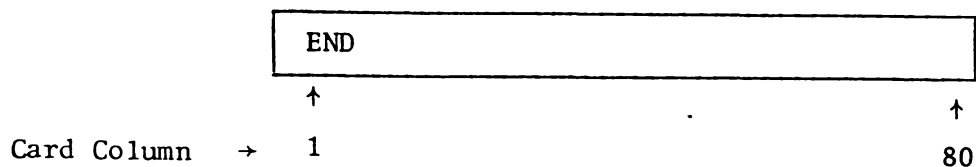
Format

DATA SUBSET COMPLETE

	↑	↑
Card Column →	1	80

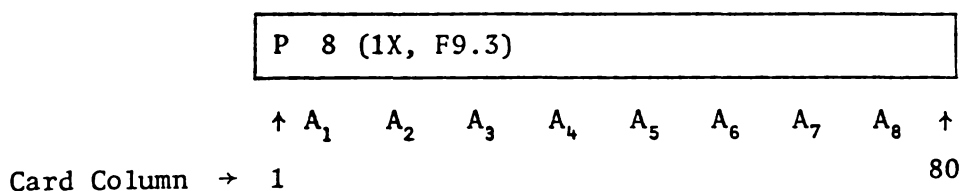
(only "D" in column 1 is necessary)

"E" indicates the end of the card input file.



(only "E" in column 1 is necessary)

"P" identifies the cards containing all the input parameters necessary to compute the catecholamine concentrations and consists of a set of 2 cards placed anywhere before the assay data in the card deck. If parameter changes are necessary during the processing of a group of samples another set of these parameter cards is inserted at the appropriate place and the changes are effected before further calculation. This situation arises for example where samples are assayed on different days or where the use of a new tracer solution has been necessary during an experiment.



where A_1 is the fractional interference by dopamine in the 1-dopa assay
 A_2 is the fractional interference by 1-dopa in the dopamine assay
 A_3 is the fractional interference by adrenaline in the noradrenaline assay
 A_4 is the fractional interference by noradrenaline in the adrenaline assay
 A_5 is the number of counts per unit time added to the original plasma sample

A_6 is the background radioactive count

A_7 volume of the extracted eluant counted (ml)

A_8 extraction eluant volume (used to compute recovery)

Card 2

Format

P	4 (2(1X, 15), F8.1)
---	---------------------

↑ I_1 I_2 P_1 I_3 I_4 P_2 I_5 I_6 P_3 I_7 I_8 P_4 ↑

Card Column → 1

80

where I_1, I_3, I_5, I_7 represent the peak heights of the instrument standard for the determination of l-dopa, dopamine, noradrenaline and adrenaline respectively.

I_2, I_4, I_6, I_8 represent the peak heights of the standard solutions of l-dopa, dopamine, noradrenaline and adrenaline respectively.

P_1, P_2, P_3, P_4 represent the concentration of the standard l-dopa, dopamine, noradrenaline and adrenaline solutions respectively.

"T" represents an optional card used to specify the graph title (maximum of 79 alphanumeric characters).

Format

T GRAPH TITLE

↑ ↑

Card Column → 1

80

"U" represents an optional card used to specify the units of measurement. The default values incorporated into the programme are "NG/ML" for all 4 catecholamines but any units up to 6 characters long may be specified.

Format

U 5X, 4 (4X, A6)

↑	unit 1	unit 2	unit 3	unit 4	↑
Card Column → 1					80

where unit 1, unit 2, unit 3 and unit 4 represent the units of measurement of l-dopa, dopamine, noradrenaline and adrenaline respectively.

Assay Input Data These cards (2 for each sample) are identified by the absence of an alphabetic character in column 1 and have the following format.

Card 1
Format

I5, 1X, I1, IX, F6.1, 1X, F8.1

↑	I_1	I_2	P_1	P_2	↑
Card Column → 1					80

I5, 1X, I1, 1X, 4(2X, 4I4)

↑	I_1	I_2	I_3	I_4	...	I_{17}	I_{18}	↑
Card Column → 1								80

where I_1 is the sample number

I_2 is the sample card number (either has the value 1 or 2)

P_1 is the sample volume

P_2 is the radioactive count of the sample

I_3, I_7, I_{11}, I_{15} are the fluorescence readings of the first duplicate for l-dopa, dopamine, noradrenaline and adrenaline respectively.

I_4, I_8, I_{12}, I_{16} are the fluorescence readings of the second duplicate for l-dopa, dopamine, noradrenaline and adrenaline respectively.

I_5 , I_9 , I_{13} , I_{17} are the fluorescence readings for the 1-dopa, dopamine, noradrenaline and adrenaline blanks respectively.

I_6 , I_{10} , I_{14} , I_{18} are the fluorescence readings of the standard solution used to monitor instrument sensitivity at the excitation and emission wavelengths used for the assay of 1-dopa, dopamine, noradrenaline and adrenaline respectively.

Normally only 2 or 3 catecholamines are determined in any one biological sample and the input fields representing unassayed catecholamines and their associated parameters are left blank. Rather than read and write assay data blanks as zero values the programme is so organized that blanks are also used in the line printer output to represent unassayed compounds. This feature requires repeat overprinting of every output line for each separate catecholamine. The result however is a less cluttered display and zero levels of assayed catecholamines are distinguished from an unassayed catecholamine. In the same manner a missing duplicate determination or an error condition arising during the analysis is output as a blank space (together with a warning label where appropriate).

Error Conditions

Extensive error checking steps have been incorporated into RXPC to detect and prevent the output of erroneous results.

Error Types

1. Duplicate negative when corrected for cross interference (duplicate eliminated).
2. Fluorimetric blank exceeds sample reading (duplicate eliminated).
3. Missing fluorimeter standard fluorescence reading (catecholamine calculation eliminated).

4. Sample numbers on an input card set differ (sample eliminated).
5. Card numbers on an input card set are incorrect (sample eliminated).
6. Blank count exceeds sample count (sample eliminated).
7. Blank count exceeds standard count (subset terminated).
8. Error in input parameter (subset terminated).
9. Error limit (25) exceeded (subset terminated).
10. Number of samples in a subset exceeds 120 (subset terminated).
11. Missing sample fluorescence readings when blank and/or instrument standard fluorescence readings are present (catecholamine calculation terminated).

Warning Types

1. Sample concentration of 1-dopa or dopamine calculated without cross-correction for interference (either because one of these compounds was not assayed or because of the presence of an error condition).
2. Sample concentration of noradrenaline or adrenaline calculated without cross-correction for interference.

Calculations

Since significant cross-interference between 1-dopa and dopamine on the one hand and noradrenaline and adrenaline on the other occurs in the fluorimetric assay (§4.4) simultaneous equations are used to correct for this effect where both members of the pair are present within the same sample. Each catecholamine is corrected for recovery from the original biological fluid or tissue by dividing the total counts in the assayed eluant by the count originally added to the sample. In this way the proportion of the catecholamine content of the biological sample in the assayed eluant is known and the original catecholamine concentration derived by dividing by the sample volume. Thus losses or

variability in the assay procedures are of little consequence since each sample is individually corrected. Small variations in the sensitivity of the fluorimeter are accounted for by the use of a fluorescent standard determined periodically during an assay series.

Equations

_____ correction for sample blank fluorescence, correction for changes in instrument sensitivity and scaling against the standard catecholamine solution fluorescence

$$X_u = \frac{P_u - B_u}{R_u} \times \frac{R_s C_s}{P_s - B_s} \quad (1)$$

_____ corrections for recovery and sample volume

$$X'_u = \frac{D_s - G}{D_u - G} \times \frac{T_u}{V} \times X_u$$

_____ correction for cross interference

$$C_u = \frac{X'_u - F_{gu} X'_g}{1 - F_{gu} F_{ug}}$$

where P_u is a sample (mean duplicate) fluorescence reading of the computed catecholamine (u)

B_u is the sample blank fluorescence

R_u is the instrument standard fluorescence

R_s is the instrument standard fluorescence associated with the catecholamine standard determination

C_s is the concentration of the catecholamine standard

P_s is the catecholamine standard fluorescence

B_s is the catecholamine standard blank

D_s is the count rate of the radioactive catecholamine added to the original biological sample

G is the background count
 D_u is the sample radioactive count
 T_u is the volume of the eluate counted
 V is the volume of the biological sample
 X'_g is the concentration of the interfering catecholamine
 (g) (uncorrected for cross-interference)
 F_{gu} is the fractional interference in the assay by the
 cross-reacting catecholamine (g)
 F_{ug} is the fractional interference by the computed
 catecholamine (u) in the assay for the cross-
 reacting catecholamine (g)

Output

An example of the output generated by RXPC is shown in Figure III-1 for the determination of the adrenaline and noradrenaline responses of lambs to electrical stunning (§7.3). The output in 3 sections, the first (Figure III-1a) containing full details of the input parameters, input assay data and any descriptive comments regarding the samples. In the next section (Figure III-1b) each individual duplicate calculation is tabulated together with means, standard deviations and an estimate of the precision.

$$\text{estimate of the SD} = \sqrt{\frac{\Sigma d^2}{2N}}$$

where Σd^2 is the sum of the squared differences between the N sets of duplicates. The value of this calculation is that information is obtained concerning the precision of the method under the conditions of each assay series and allows trends to be monitored and action to be taken when the estimate of the standard deviation exceeds previous limits (Brown *et al.* 1957). Finally the catecholamine concentrations are plotted against sample number (Figure III-1c).

CATECHOLAMINE LEVELS IN LAMBS ELECTRICALLY STUNNED (-1 MINUTE TO +2 HOURS)

RUAKURA ABATTOIR 30-5-77

SAMPLES 1608-1616 LAMP M730 1ST STUN
 SAMPLES 1617-1625 LAMP M795 1ST STUN
 SEE SECTION 7.3 FOR FURTHER DETAILS

*****PARAMETER INPUT***

A(1)	A(2)	A(3)	A(4)	A(5)	A(6)	A(7)	A(8)
0.000	0.000	0.120	0.021	5550.200	28.500	2.760	.13
		B(1)	B(2)	B(3)	B(4)		
		0.000	0.000	7.500	15.882		

ASSAY INPUT DATA

NO	VOL	COUNT	L-DOPA	DOPAMINE	NORADRENALINE				ADRENALINE			
1608	3.6	590.0			50	49	47	33	43	45	43	36
1609	3.6	571.2			336	346	50	33	254	262	45	36
1610	3.0	602.9			124	125	48	33	130	146	43	36
1611	3.8	724.8			75	74	50	33	69	75	46	36
1612	3.8	588.9			60	60	52	33	48	48	45	33
1613	3.8	629.9			55	57	48	33	44	45	42	33
1614	3.6	540.8			62	62	48	33	50	51	45	33
1615	3.2	640.4			56	57	48	33	48	48	45	33
1616	4.6	604.1			53	51	48	33	46	46	44	33
1617	5.4	631.1			53	53	50	33	46	45	45	33
1618	5.0	577.1			273	267	60	36	363	377	44	33
1619	4.0	636.9			85	88	50	36	138	147	45	33
1620	4.0	647.5			74	72	50	36	65	65	45	33
1621	5.4	626.4			64	63	53	36	60	62	40	36
1622	5.2	634.6			64	68	50	36	59	66	44	36
1623	4.2	704.9			77	78	56	36	63	60	47	36
1624	4.4	618.2			47	47	44	36	40	40	32	36
1625	4.4	639.3			53	53	47	36	40	40	33	36

Figure III-1a. Input data for computer programme RXPC

CALCULATED CATECHOLAMINE CONCENTRATIONS AND RECOVERY

SAMPLE NO	RECOVERY %	CHECKS	L-DOPA NG/ML	DOPAMINE NG/ML	NORADRENALINE NG/ML	ADRENALINE NG/ML			
1608	47.9				0.4 0.2 0.3	0.7 0.7 0.7			
1609	46.3				58.9 40.6 39.8	74.2 73.5 73.8			
1610	49.0				9.5 9.7 9.6	41.5 40.0 40.8			
1611	59.4				2.5 2.2 2.2	5.8 7.5 6.6			
1612	47.8				1.1 1.1 1.1	1.0 1.0 1.0			
1613	51.3				0.9 1.2 1.1	0.6 0.9 0.7			
1614	43.7				2.2 2.2 2.2	1.8 2.2 2.0			
1615	52.2				1.2 1.4 1.5	1.0 1.0 1.0			
1616	49.1				0.6 0.3 0.4	0.5 0.5 0.5			
1617	51.4				0.3 0.3 0.3	0.2 0.0 0.1			
1618	46.8				14.8 14.1 14.5	80.6 83.7 84.1			
1619	51.9				1.2 1.6 1.4	26.4 29.0 27.7			
1620	52.8				2.4 2.2 2.3	5.5 5.5 5.5			
1621	51.0				0.8 0.7 0.7	2.5 2.7 2.5			
1622	51.7				0.9 1.3 1.1	3.2 4.7 3.9			
1623	57.7				1.9 2.1 2.0	3.7 3.0 3.4			
1624	50.3				0.1 0.1 0.1	2.1 2.1 2.1			
1625	52.1				0.5 0.5 0.5	1.7 1.7 1.7			
MEAN	50.7				4.5 4.5 4.5	14.0 14.4 14.2			
S.D.	3.8				9.4 9.7 9.5	25.4 25.7 25.6			
NO	18				18 18 18	18 18 18			
MEAN DIF BETWEEN DUPLICATES									
S.D.									
NO									
ESTIMATE OF PRECISION									

Figure III-1b. Catecholamine concentrations
calculated by computer programme
RXPC

CATECHOLAMINE LEVELS IN LAMBS ELECTRICALLY STUNNED 30-3-77

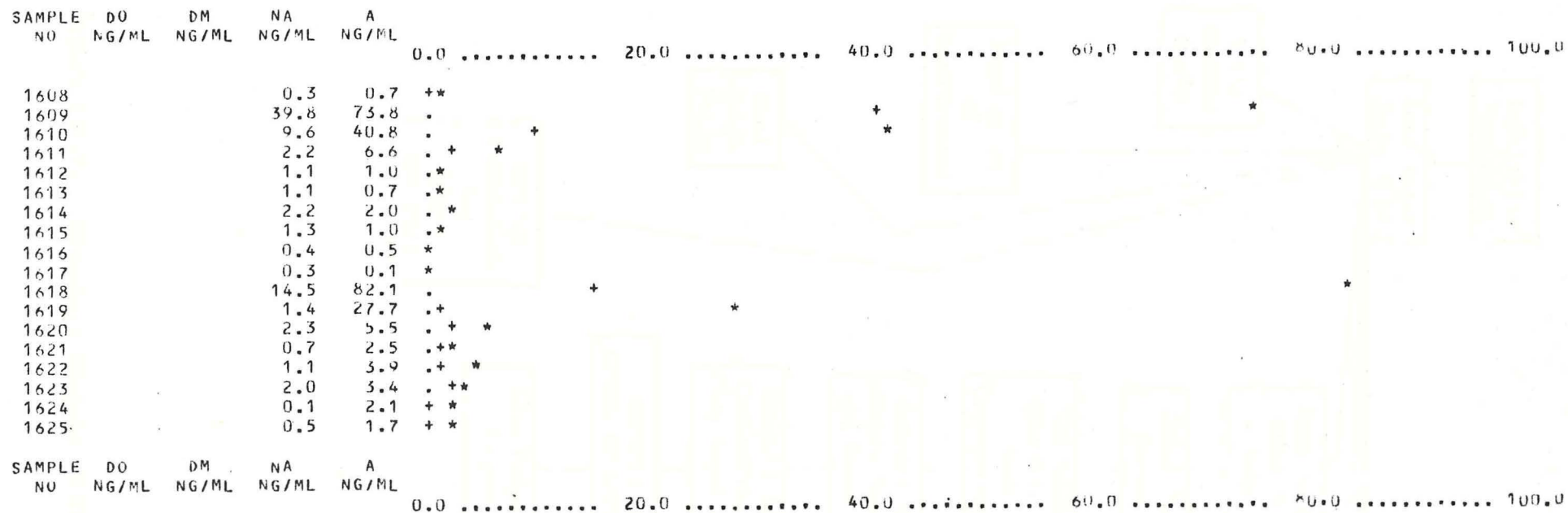


Figure III-1c. Catecholamine concentrations graphed by computer programme RXPC

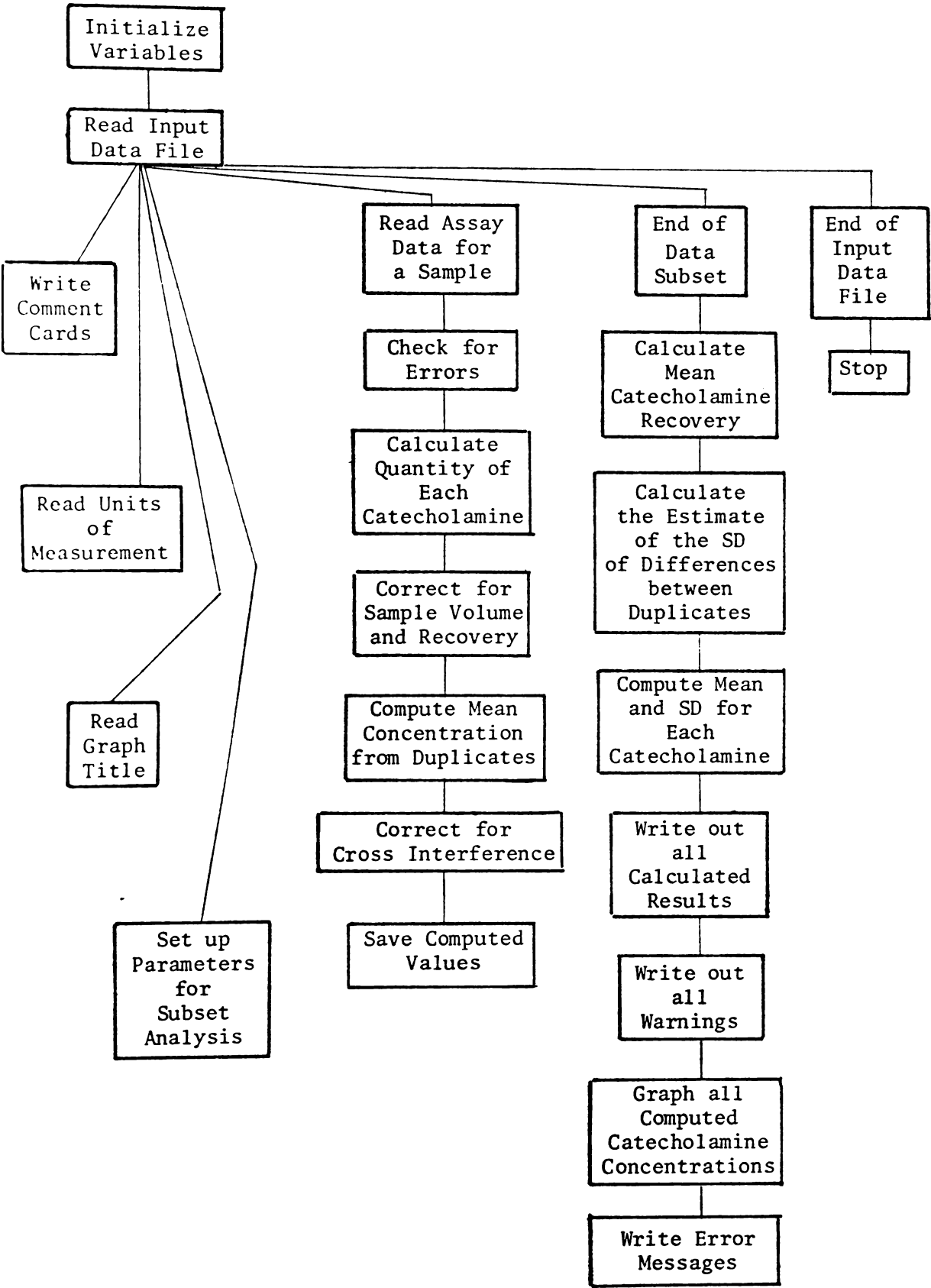


Figure III-2. Structure Diagram of RXPC

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